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(57) Abstract

Pharmaceutical compostions comprising liposomes containing antisense oligonucleotides are provided for the modulation of expression of the human ras gene in both the normal (wildtype) and activated (mutant) forms.

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LIPOSOMAL OLIGONUCLEOTIDE COMPOSITIONS FOR MODULATING ras GENE EXPRESSION

FIELD OF THE INVENTION

This invention relates to pharmaceutical compositions comprising liposomes containing one or more antisense oligonucleotides. The antisense oligonucleotides contained within liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human wildtype or mutant ras sequence and are capable, individually and/or collectively, of modulating ras expression. In another embodiment, the liposomes of the invention contain (a) one or more such antisense oligonucleotides and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism.

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cellular growth (proliferation) and differentiation are considered to be causative events leading to the development of tumors and cancers (see, generally, Weinberg, *Sci. American* 275:62, 1996). There are many families of genes presently implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal cell growth and maturation. Mutation

of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "activated." It is thought that such a point mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras genes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, K-ras and N-ras).

It is presently believed that inhibiting expression of activated cancer-associated genes in a particular tumor cell might force the cell back into a more normal growth habit. For example, Feramisco *et al.* (*Nature, 314*:639, 1985) demonstrated that cells transformed to a malignant state with an activated ras gene slow their rate of proliferation and adopt a more normal appearance when microinjected with an antibody which binds to the protein product of the ras gene. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of cancer cells.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a

particular variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

There is a great desire to provide compositions of matter which can modulate the expression of the ras gene, and particularly to provide compositions of matter which specifically modulate the expression of the activated form of the ras gene. Inhibition of K-ras gene expression has been accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the K-ras gene RNA in antisense orientation (Mukhopadhyay *et al.*, *Cancer Research 51*:1744, 1991; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges *et al.*, *Cancer Research*, *53*:1743, 1993).

Antisense oligonucleotide inhibition of expression has proven to be a useful tool in understanding the role(s) of various cancer-associated gene families. Antisense oligonucleotides are small oligonucleotides which are complementary to the "sense" (coding strand) of a given gene, and are thus also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt *et al.* (*Mol. Cell Biol. 8*:963, 1988) state that antisense oligonucleotides designed to hybridize specifically with (*i.e.*, "targeted to") mRNA transcripts of the c-myc gene inhibit proliferation and induce differentiation when added to cultured HL60 leukemic cells. Anfossi *et al.* (*Proc. Natl. Acad. Sci. 86*:3379, 1989) state that antisense oligonucleotides targeted to the c-myb gene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom *et al.* (*Proc. Nat. Acad. Sci. 85*:1028, 1988) state that expression of the protein product of the c-myc gene and proliferation of HL60 cultured leukemic cells are both inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA.

With specific regard to oligonucleotides having ras sequences, United States Patent No. 4,871,838 to Bos *et al.* discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect this mutation. Helene and co-workers have reported the selective inhibition of activated (codon 12 G→T transition) H-ras mRNA expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail; this compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations (Saison-Behmoaras *et al.*, *EMBO J. 10*:1111, 1991). Chang *et al.* (*Biochemistry 30*:8283, 1991) disclose selective targeting of a mutant H-ras message, specifically, H-ras codon 61 containing an A-T transversion, with an 11-mer methylphosphonate oligonucleotide or its psoralen derivative. These compounds, which required concentrations of 7.5-150 μM for activity, were shown by immunoprecipitation to selectively inhibit mutant $p21^{H\text{-}ras}$ expression relative to wildtype $p21^{H\text{-}ras}$.

Although it has been recognized that antisense oligonucleotides have great therapeutic potential, there remains a long-felt need for pharmaceutical compositions and methods that could positively alter the *in vivo* stability, concentration, and distribution of such oligonucleotides. Enhanced biostability of antisense oligonucleotides in a mammal would generally be preferred for improved delivery of the oligonucleotide to its intended target tissue(s) with potentially less frequent dosing. For antisense oligonucleotides targeted to oncogenic molecules, enhanced distribution to tumor tissues would be preferred.

OBJECTS OF THE INVENTION

It is an object of the invention to provide liposomes containing one or more antisense oligonucleotides and pharmaceutical compositions comprising such liposomes, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human ras sequence and are capable, either individually or collectively, of modulating ras expression.

It is another object of the invention to provide liposomes containing one or more antisense oligonucleotides, and pharmaceutical compositions comprising such liposomes, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding an activated (mutant) human ras sequence and are capable, either individually or collectively, of modulating the expression of the activated form of the ras gene.

It is a further object of the invention to provide liposomes containing (a) one or more antisense oligonucleotides being from about 8 to about 30 nucleotides in length, targeted to a nucleic acid encoding either a wildtype or mutant human ras sequence which are capable, either individually or collectively, of modulating ras expression and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism.

An additional object of the invention is to provide liposome-based pharmaceutical compositions which inhibit the hyperproliferation of cells, including cancerous cells. Methods of inhibiting the hyperproliferation of cells, including cancerous cells, are also an object of this invention.

A further object of this invention is to provide methods of treatment of, and liposome-based pharmaceutical

compositions for, conditions arising due to mutation of the gene from the wildtype to a mutant, activated form of the ras gene.

SUMMARY OF THE INVENTION

In accordance with the present invention liposomes containing one or more antisense oligonucleotides and pharmaceutical compositions comprising such liposomes are provided, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human ras sequence and are capable, either individually or collectively, of modulating ras expression.

Also provided are liposomes containing one or more antisense oligonucleotides, and pharmaceutical compositions comprising such liposomes, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding an activated (mutant) human ras sequence and are capable, either individually or collectively, of modulating the expression of the activated form of the ras gene.

Further provided are liposomes containing (a) one or more antisense oligonucleotides being from about 8 to about 30 nucleotides in length, targeted to a nucleic acid encoding either a wildtype or mutant human ras sequence which are capable, either individually or collectively, of modulating ras expression and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism.

Liposome-based pharmaceutical compositions which inhibit the hyperproliferation of cells, including cancerous cells, are provided. Methods of inhibiting the hyperproliferation of cells, including cancerous cells, are also provided.

Methods of treatment of, and liposome-based pharmaceutical compositions for, conditions arising due to mutation of the gene from the wildtype to a mutant, activated form of the ras gene are also provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the time course of the clearance of ISIS 2503 and oligonucleotide metabolites from blood. Each point is represented as the mean (symbol) ± standard deviation (n=1-8). Nonlinear regression was performed using a one compartment model (solid line). Symbols: ●, observed blood concentration for liposomal oligonucleotide formulation; the solid line indicates the predicted blood concentration for liposomal oligonucleotide formulation; ▲, observed blood concentration for saline formulation of ISIS 2503.

Figure 2 shows the distribution and kinetics of full-length ISIS 2503 in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, axillary and inguinal lymph nodes, combined; ▲, mesenteric and mandibular lymph nodes, combined; ●, hand skin; ♦, back skin.

Figure 3 shows the distribution and kinetics of full-length ISIS 2503 in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, liver; ▲, spleen; ●, kidney cortex; ♦, kidney medulla.

Figure 4 shows the distribution and kinetics of total oligonucleotide in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, axillary and inguinal lymph nodes, combined; ▲, mesenteric and mandibular lymph nodes, combined; ●, hand skin; ♦, back skin.

Figure 5 shows the distribution and kinetics of total oligonucleotide in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, liver; ▲, spleen; ●, kidney cortex; ♦, kidney medulla.

Figures 6A and 6B are representative electropherograms of a liposomal (Figure 6A) or saline formulation (Figure 6B) of ISIS 2503 in monkey blood samples. Samples were taken 60 hours (a) after an 0.5 hour infusion or (b) 1 hour after initiation of a 2-hour infusion of 10 mg/kg of the respective formulations. Peaks corresponding to ISIS 2503 and various metabolites (arrows) or to an internal standard (T_{27}, a 27-mer phosphorothioate oligodeoxythymidine) are indicated.

Figures 7A and 7B are representative electropherograms of a liposomal (Figure 7A) or saline formulation (Figure 7B) of ISIS 2503 in monkey kidney cortex samples. Samples were taken 60 hours (a) after an 0.5 hour infusion or (b) 48 hours after the last 2-hour infusion of 14 total doses administered every other day of 10 mg/kg of the respective formulations. Peaks corresponding to ISIS 2503 and

various metabolites (arrows), including a suspected (n+1) species (see Examples), or to an internal standard (T_{27}, a 27-mer phosphorothioate oligodeoxythymidine) are indicated.

DETAILED DESCRIPTION OF THE INVENTION

Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control characteristic of cancer cells, *i.e.*, continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell transformation in carefully controlled, quantitative *in vitro* assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-associated genes are believed to acquire transformation-inducing properties through mutations leading to changes in the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of cancer associated genes, resulting in over- or under-expression of their gene products. In other cases, gene mutations occur within the coding regions of cancer associated genes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

Many cellular cancer associated gene families have been identified and categorized on the basis of their subcellular location and the putative mechanism of action of their protein products. The ras genes are members of a gene

family which encode related proteins that are localized to the inner face of the plasma membrane. Ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity (for a review, see Downward, *Trends Biochem. Sci. 15*:469, 1990). Although their cellular function(s) is(are) unknown, the biochemical properties of the ras proteins, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes. The ras gene product, $p21^{ras}$, interacts with a variety of known and proposed cellular effectors (for a review, see Marshall, *Trends Biochem. Sci. 18*:250, 1993)

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras genes have been localized to codons 12, 13, and 61. The sequences of H-ras, K-ras and N-ras are known (Capon *et al.*, *Nature 302*:33, 1983; Kahn *et al.*, *Anticancer Res.* 7:639, 1987; Hall and Brown, *Nucleic Acids Res. 13*:5255, 1985). The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product (Tabin *et al.*, *Nature, 300*:143, 1982; Reddy *et al.*, *Nature 300*:149, 1982; Taparowsky *et al.*, *Nature 300*:762, 1982). This single amino acid change is thought to abolish normal control and/or function of $p21^{H\text{-}ras}$, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such

deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The present invention provides pharmaceutical compositions comprising liposomes containing one or more antisense oligonucleotides, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, more preferably from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human wildtype or mutant ras sequence and are capable, individually and/or collectively, of modulating ras expression. In another embodiment, compositions of the invention comprise sterically stabilized liposomes containing (a) one or more such antisense oligonucleotides and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism. The remainder of the Detailed Description relates in more detail to (1) the oligonucleotides of the invention, (2) their bioequivalents, (3) sterically stabilized liposomes, (4) chemotherapeutic agents that can be combined with antisense oligonucleotides targeted to H-ras in the context of the liposomes of the invention and (5) administration of pharmaceutical compositions comprising the liposomal oligonucleotide compositions of the invention.

1. Oligonucleotides: In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

An oligonucleotide is a polymer of a repeating unit generically known as a nucleotide. The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogen-containing heterocyclic *base* linked by one of its nitrogen atoms to (2) a 5-pentofuranosyl *sugar* and (3) a *phosphate* esterified to one of the 5' or 3' carbon atoms of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to an adjacent sugar of a second, adjacent nucleotide via a 3'-5' phosphate linkage. The "backbone" of an unmodified oligonucleotide consists of (2) and (3), that is, sugars linked together by phosphodiester linkages between the 5' carbon of the sugar of a first nucleotide and the 3' carbon of a second, adjacent nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems (Foster City, CA). Any other means for such synthesis may also be employed, however, the actual synthesis of the oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides which specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. An oligonucleotide is specifically hybridizable to its target sequence due to the formation of base pairs between specific partner nucleobases in the interior of a nucleic acid duplex. Among the naturally occurring nucleobases, guanine (G) binds to cytosine (C), and adenine (A) binds to thymine (T) or uracil (U). In addition to the equivalency of U (RNA) and T (DNA) as partners for A, other naturally occurring nucleobase equivalents are known, including 5-methylcytosine, 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC (C equivalents), and 5-hydroxymethyluracil (U equivalent). Furthermore, synthetic

nucleobases which retain partner specificity are known in the art and include, for example, 7-deaza-Guanine, which retains partner specificity for C. Thus, an oligonucleotide's capacity to specifically hybridize with its target sequence will not be altered by any chemical modification to a nucleobase in the nucleotide sequence of the oligonucleotide which does not significantly effect its specificity for the partner nucleobase in the target oligonucleotide. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The nucleotide sequences of the oligonucleotides of the invention are given in Example 1 and also in the Sequence Listing. Citations for target H-ras sequences are also presented in Example 1.

Antisense oligonucleotides are commonly used as research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses.

A. Modified Linkages: Specific examples of some preferred modified oligonucleotides envisioned for this

invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioates and those with CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2 [known as a methylene(methylimino) or MMI backbone], CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 and O-N(CH_3)-CH_2-CH_2 backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2). Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent No. 5,034,506). Further preferred are oligonucleotides with NR-C(*)-CH_2-CH_2, CH_2-NR-C(*)-CH_2, CH_2-CH_2-NR-C(*), C(*)-NR-CH_2-CH_2 and CH_2=C(*)-NR-CH_2 backbones, wherein "*" represents O or S (known as amide backbones; DeMesmaeker *et al.*, WO 92/20823, published November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al.*, *Science*, 1991, *254*:1497; U.S. Patent No. 5,539,082).

B. Modified Nucleobases: The oligonucleotides of the invention may additionally or alternatively include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, *e.g.*, hypoxanthine, 6-methyladenine, 5-methylcytosine, 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC, as well synthetic nucleobases, *e.g.*, 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6(6-aminohexyl)adenine and 2,6-diaminopurine (Kornberg, A., *DNA Replication*, W.H. Freeman &

Co., San Francisco, 1980, pages 75-77; Gebeyehu, G., *et al.*, *Nucleic Acids Res.*, 1987, *15*, 4513).

C. Sugar Modifications: The oligonucleotides of the invention may additionally or alternatively comprise substitutions of the sugar portion of the individual nucleotides. For example, oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other preferred modified oligonucleotides may contain one or more substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, OCH_3OCH_3, $OCH_3O(CH_2)_nCH_3$, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl)] (Martin *et al.*, *Helv. Chim. Acta*, 1995, *78*:486). Other preferred modifications include 2'-methoxy- (2'-O-CH_3), 2'-propoxy- (2'-$OCH_2CH_2CH_3$) and 2'-fluoro- (2'-F).

D. Other Modifications: Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. The 5' and 3' termini of an oligonucleotide may also be modified to serve as points of chemical conjugation of, *e.g.*, lipophilic moieties (see immediately subsequent paragraph), intercalating agents (Kuyavin *et al.*, WO 96/32496, published

October 17, 1996; Nguyen *et al.*, U.S. Patent No. 4,835,263, issued May 30, 1989) or hydroxyalkyl groups (Helene *et al.*, WO 96/34008, published October 31, 1996).

Other positions within an oligonucleotide of the invention can be used to chemically link thereto one or more effector groups to form an oligonucleotide conjugate. An "effector group" is a chemical moiety that is capable of carrying out a particular chemical or biological function. Examples of such effector groups include, but are not limited to, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A variety of chemical linkers may be used to conjugate an effector group to an oligonucleotide of the invention. As an example, U.S. Patent No. 5,578,718 to Cook *et al.* discloses methods of attaching an alkylthio linker, which may be further derivatized to include additional groups, to ribofuranosyl positions, nucleosidic base positions, or on internucleoside linkages. Additional methods of conjugating oligonucleotides to various effector groups are known in the art; see, *e.g.*, *Protocols for Oligonucleotide Conjugates (Methods in Molecular Biology, Volume 26)* Agrawal, S., ed., Humana Press, Totowa, NJ, 1994.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N^6 position

of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., *et al., Nucleic Acids Res.*, 1987, *15:*4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger *et al., Proc. Natl. Acad. Sci. U.S.A.*, 1989, *86:*6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Let.*, 1994, *4:*1053), a thioether, *e.g.*, hexyl-*S*-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.*, 1992, *660:*306; Manoharan *et al., Bioorg. Med. Chem. Let.*, 1993, *3:*2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.*, 1992, *20:*533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al., EMBO J.*, 1991, *10:*111; Kabanov *et al., FEBS Lett.*, 1990, *259:*327; Svinarchuk *et al., Biochimie*, 1993, *75:*49), a phospholipid, *e.g.*, di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.*, 1995, *36:*3651; Shea *et al., Nucl. Acids Res.*, 1990, *18:*3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides*, 1995, *14:*969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.*, 1995, *36:*3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta*, 1995, *1264:*229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al., J. Pharmacol. Exp. Ther.*, 1996, *277:*923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, are disclosed in co-owned U.S. Patents Nos. 5,138,045, 5,218,105 and 5,459,255.

The present invention also includes oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (see co-owned U.S. Patent No. 5,587,361 to Cook *et al.*) and those having substantially

chirally pure (Sp or Rp) alkylphosphonate, phosphoamidate or phosphotriester linkages (see co-owned U.S. Patents Nos. 5,212,295 and 5,521,302).

E. Chimeric Oligonucleotides: The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. By way of example, such "chimeras" may be "gapmers," *i.e.*, oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for the target RNA molecule but are unable to support nuclease activity (*e.g.*, 2'-fluoro- or 2'-methoxyethoxy- substituted). Other chimeras include "wingmers," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for, *e.g.*, RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is

unable to support nuclease activity (*e.g.*, 2'-fluoro- or 2'-methoxyethoxy- substituted), or *vice-versa*.

F. Synthesis: The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

1. Teachings: regarding the synthesis of particular modified oligonucleotides may be found in the following U.S. patents or pending patent applications, each of which is commonly assigned with this application and is hereby incorporated by reference: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,30712, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having b-lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to

nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patents Nos. 5,223,168, issued June 29, 1993, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, *inter alia*, methods of synthesizing 2'-fluoro-oligonucleotides. In 2'-methoxyethoxy-modified oligonucleotides, 5-methyl-2'-methoxyethoxy-cytosine residues are used and prepared as described in pending application Serial No. 08/731,199, filed October 4, 1996. Specific methods for preparing MMI linkages are taught in United States Patent Nos. 5,378,825 (issued January 3, 1995), 5,386,023 (issued January 31, 1995), 5,489,243 (issued on February 6, 1996), 5,541,307 (issued on July 30, 1996), 5,618,704 (issued April 8, 1997) and 5,623,070 (issued April 22, 1997). MMI is an abbreviation for methylene(methylimino) that in turn is a shorten version of the more complex chemical nomenclature "3'-de(oxyphosphinico)-3'[methylene(methylimino)]." Irrespective of chemical nomenclature, the linkages are as described in these patents. The linkages of these patents have also been described in various scientific publications by the inventors and their co-authors including Bhat *et al.* (*J. Org. Chem. 61*:8186, 1996, and references cited therein).

2. Bioequivalents: The compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to "prodrugs" and "pharmaceutically acceptable salts" of the oligonucleotides of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

A. Oligonucleotide Prodrugs:

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993.

B. Pharmaceutically Acceptable Salts: The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligonucleotides of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline,

diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, *66*:1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid,

methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation including, for example, alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

3. Sterically Stabilized Liposomes: In compositions of the invention, one or more antisense oligonucleotides and/or therapeutic agents are entrapped within liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see,

generally, Chonn *et al.*, *Current Op. Biotech.* *6*:698, 1995). The therapeutic potential of liposomes as drug delivery agents was recognized nearly thirty years ago (Sessa *et al.*, *J. Lipid Res.* *9*:310, 1968). Liposomes include "sterically stabilized liposome," a term which, as used herein, refers to a liposome comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters* *223*:42, 1987; Wu *et al.*, *Cancer Research* *53*:3765, 1993).

A. Glycolipid-comprising liposomes: Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, *507*:64, 1987) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* *85*:6949, 1988). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-

sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

B. Liposomes derivatized with hydrophilic polymers: Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn. 53*:2778, 1980) described liposomes comprising a nonionic detergent, 2C$_{12}$15G, that contains a PEG moiety. Illum *et al.* (*FEBS Letters 167*:79, 1984) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Letts. 268*:235, 1990) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta 1029*:91, 1990) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos.

5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

C. Liposomes comprising nucleic acids: A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

4. Chemotherapeutic Agents: Certain embodiments of the invention provide for liposomes containing (a) one or more antisense oligonucleotides targeted to a nucleic acid encoding a ras protein and (b) one or more chemotherapeutic agents which do not function by an antisense mechanism. In a related embodiment, such chemotherapeutic agents are co-administered with one or more of the liposomal oligonucleotide compositions of the invention but are separately encapsulated in distinct liposomes or are administered by a non-liposomal delivery mechanism. As used herein, a "chemotherapeutic agent" is an anticancer agents that functions via a conventional (*i.e.*, non-antisense) mode of action. Examples of such chemotherapeutic agents include, but are not limited to, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow *et*

al., eds., 1987, Rahay, N.J., pages 1206-1228). When used with the liposomal oligonucleotide compositions of the invention, such chemotherapeutic agents may be used individually, sequentially (*e.g.*, 5-FU for a period of time followed by MTX), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU and MTX).

In a related embodiment, liposomes containing (a) one or more antisense oligonucleotides targeted to a first nucleic acid encoding a ras sequence and (b) one or more additional antisense oligonucleotides targeted to a second nucleic acid encoding a cancer associated gene. By the term "cancer associated gene" is intended any cellular or viral gene the expression of which disrupts regulation of the cell cycle, negatively effects contact inhibition of growth, leads to cellular hyperproliferation, promotes pre-metastatic or metastatic events and/or otherwise leads to cellular hyperproliferation, tumor formation and the growth and spread of cancers, regardless of mechanism of action. In a related embodiment, such additional antisense oligonucleotides targeted to a second cancer-associated gene are co-administered with one or more of the liposomal oligonucleotide compositions of the invention but are separately encapsulated in distinct liposomes or are administered by a non-liposomal delivery mechanism. Such antisense oligonucleotides targeted to a second cancer associated gene include, but are not limited to, those directed to the following targets as disclosed in the indicated co-owned U.S. Patents, pending applications or published PCT applications, which are hereby incorporated by reference: raf (WO 96/39415, WO 95/32987 and U.S. Patent Nos. 5,563,255, issued October 8, 1996, and 5,656,612, issued August 12, 1997), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent No. 5,656,743, issued August 12, 1997), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO

95/10938 and U.S. Patent No. 5,510,239, issued March 23, 1996), subunits of transcription factor AP-1 (co-pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (co-pending application U.S. Serial No. 08/910,629, filed August 13, 1997), and MDR-1 (multidrug resistance glycoprotein; co-pending application U.S. Serial No. 08/731,199, filed September 30, 1997).

5. Administration of Pharmaceutical Compositions: The formulation of pharmaceutical compositions comprising the liposomal oligonucleotide compositions of the invention and their subsequent administration is believed to be within the skill of those in the art. In general, for therapeutics, a patient in need of such therapy is administered a liposomal oligonucleotide composition in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01 μg to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for

changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the oligonucleotide may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

An optimal dosing schedule is used to deliver a therapeutically effective amount of the oligonucleotide being administered via a particular mode of administration. The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of oligonucleotide-containing pharmaceutical composition which is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a pharmaceutical composition, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

In some cases it may be more effective to treat a patient with a liposomal oligonucleotide composition of the invention in conjunction with other, traditional therapeutic modalities in order to increase the efficacy of a treatment

regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the pharmaceutical composition may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, *e.g.*, individual or family history or genetic testing, that there is a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. As part of a treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have compared to the effect observed when a second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Typically, parenteral administration is employed. The term "parenteral delivery" refers to the administration of an oligonucleotide of the invention to an

animal in a manner other than through the digestive canal. Parenteral administration includes intravenous (i.v.) drip, subcutaneous, intraperitoneal (i.p.) or intramuscular injection, or intrathecal or intraventricular administration. Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Means of preparing and administering parenteral pharmaceutical compositions are known in the art (see, *e.g.*, Avis, Chapter 84 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1545-1569). Parenteral means of delivery include, but are not limited to, the following illustrative examples.

(A) Intravitreal injection, for the direct delivery of drug to the vitreous humor of a mammalian eye, is described in U.S. Patent No. 5,591,720, the contents of which are hereby incorporated by reference. Means of preparing and administering ophthalmic preparations are known in the art (see, *e.g.*, Mullins *et al.*, Chapter 86 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1581-1595).

(B) Intravenous administration of antisense oligonucleotides to various non-human mammals has been described by Iversen (Chapter 26 *In: Antisense Research and Applications*, Crooke *et al.*, eds., CRC Press, Boca Raton, FL, 1993, pages 461-469). Systemic delivery of oligonucleotides to non-human mammals via intraperitoneal means has also been described (Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 91*:11766, 1994).

(C) Intraluminal drug administration, for the direct delivery of drug to an isolated portion of a tubular organ or tissue (*e.g.*, such as an artery, vein, ureter or urethra), may be desired for the treatment of patients with diseases or conditions afflicting the lumen of such organs or

tissues. To effect this mode of oligonucleotide administration, a catheter or cannula is surgically introduced by appropriate means. For example, for treatment of the left common carotid artery, a cannula is inserted thereinto *via* the external carotid artery. After isolation of a portion of the tubular organ or tissue for which treatment is sought, a composition comprising the oligonucleotides of the invention is infused through the cannula or catheter into the isolated segment. After incubation for from about 1 to about 120 minutes, during which the oligonucleotide is taken up by cells of the interior lumen of the vessel, the infusion cannula or catheter is removed and flow within the tubular organ or tissue is restored by removal of the ligatures which effected the isolation of a segment thereof (Morishita *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 90*:8474, 1993). Antisense oligonucleotides may also be combined with a biocompatible matrix, such as a hydrogel material, and applied directly to vascular tissue *in vivo* (Rosenberg *et al.*, U.S. Patent No. 5,593,974, issued January 14, 1997).

(D) Intraventricular drug administration, for the direct delivery of drug to the brain of a patient, may be desired for the treatment of patients with diseases or conditions afflicting the brain. To effect this mode of oligonucleotide administration, a silicon catheter is surgically introduced into a ventricle of the brain of a human patient, and is connected to a subcutaneous infusion pump (Medtronic Inc., Minneapolis, MN) that has been surgically implanted in the abdominal region (Zimm *et al.*, *Cancer Research 44*:1698, 1984; Shaw, *Cancer 72(11 Suppl.)*:, 3416, 1993). The pump is used to inject the oligonucleotides and allows precise dosage adjustments and variation in dosage schedules with the aid of an external programming device. The reservoir capacity of the pump is 18-20 mL and infusion rates may range from 0.1 mL/h to 1 mL/h. Depending on the frequency

of administration, ranging from daily to monthly, and the dose of drug to be administered, ranging from 0.01 µg to 100 g per kg of body weight, the pump reservoir may be refilled at 3-10 week intervals. Refilling of the pump is accomplished by percutaneous puncture of the pump's self-sealing septum.

(E) Intrathecal drug administration, for the introduction of a drug into the spinal column of a patient may be desired for the treatment of patients with diseases of the central nervous system (CNS). To effect this route of oligonucleotide administration, a silicon catheter is surgically implanted into the L3-4 lumbar spinal interspace of a human patient, and is connected to a subcutaneous infusion pump which has been surgically implanted in the upper abdominal region (Luer and Hatton, *The Annals of Pharmacotherapy 27*:912, 1993; Ettinger *et al. Cancer, 41*:1270, 1978; Yaida *et al., Regul. Pept. 59*:193, 1985). The pump is used to inject the oligonucleotides and allows precise dosage adjustments and variations in dose schedules with the aid of an external programming device. The reservoir capacity of the pump is 18-20 mL, and infusion rates may vary from 0.1 mL/h to 1 mL/h. Depending on the frequency of drug administration, ranging from daily to monthly, and dosage of drug to be administered, ranging from 0.01 µg to 100 g per kg of body weight, the pump reservoir may be refilled at 3-10 week intervals. Refilling of the pump is accomplished by a single percutaneous puncture to the self-sealing septum of the pump. The distribution, stability and pharmacokinetics of oligonucleotides within the CNS are followed according to known methods (Whitesell *et al., Proc. Natl. Acad. Sci. U.S.A. 90*:4665, 1993).

To effect delivery of oligonucleotides to areas other than the brain or spinal column *via* this method, the silicon catheter is configured to connect the subcutaneous infusion pump to, *e.g.*, the hepatic artery, for delivery to the liver

(Kemeny *et al.*, *Cancer 71*:1964, 1993). Infusion pumps may also be used to effect systemic delivery of oligonucleotides (Ewel *et al.*, *Cancer Research 52*:3005, 1992; Rubenstein *et al.*, *J. Surg. Oncol. 62*:194, 1996).

EXAMPLES

The following examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of the present invention.

EXAMPLE 1: Nucleic Acid Sequences

The oligonucleotides of this invention are designed to be complementary to, and thus hybridizable with, messenger RNA derived from a ras gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a loss of its function in the cell. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to interfere with expression of the ras gene. Some oligonucleotides of this invention are designed to activate RNAse H cleavage of the ras mRNA.

The protein products of the other mammalian ras genes, N-ras and K-ras, are identical to H-ras over the first 85 amino acids. However, the nucleic acid sequences of the three ras genes are not identical, and persons of ordinary

skill in the art will be able to use this invention as a guide in preparing oligonucleotides specifically hybridizable with a particular ras gene. While one preferred embodiment of the invention relate to antisense oligonucleotides specifically hybridizable with codon 12 of the H-ras mRNA, the disclosure can be used by persons skilled in the art as a guide in preparing oligonucleotides specifically hybridizable with other point mutations of the H-ras gene, particularly the well defined point mutations at codon 12, codon 13 and codon 61 of H-ras, or point mutations within other ras genes.

The nucleotide sequence of wildtype (wt) H-ras, also known as Ha-ras, has been described by Capon *et al.* (*Nature 302*:33, 1983), Fasano *et al.* (*J. Mol. Appl. Genet. 2*:173, 1983), Reddy (*Science 220*:1061, 1983) and Honkawa *et al.* (*Mol. Cell. Biol.* 7:2933, 1987). Mutant (activated) H-ras sequences have been reported by Tabin *et al.* (Nature 300:143, 1982), Taparowsky *et al.* (Nature 300:762, 1982), Yuasa *et al.* (Nature 303:775, 1983), Sekiya *et al.* (*Proc. Natl. Acad. Sci. USA 81*:4771, 1984; *Jpn. J. Cancer Res. 76*:787, 1985), Kraus *et al.* (*Proc. Natl. Acad. Sci. USA 81*:5384, 1984), Stevens *et al.* (*Proc. Natl. Acad. Sci. USA 85*:3875), Deng *et al.* (*Cancer Res. 47*:3195, 1987), Santos *et al.* (*Proc. Natl. Acad. Sci. USA 80*:4679, 1983), Tanci *et al.* (*Nucleic Acids Res. 20*:1157, 1992) and Tadokoro *et al.* (*Oncogene 4*:499, 1989). The sequences of wildtype and mutant H-ras genes may also be found in the Genbank and EMBOL databases under Accession Nos. J00206, J00276, J00277, K00654, K00954, M30539, M19990, M17232, M25876, V00574, X01227 and X16438.

The nucleotide sequence of wildtype (wt) K-ras, also known as Ki-ras, has been described by McGrath *et al.* (Nature 304:501, 1983) and McCoy *et al.* (*Mol. Cell. Biol. 4*:1577, 1984). Mutant (activated) K-ras sequences have been reported by Shimizu *et al.* (*Nature 304*:497, 1983), Capon *et al.* (*Nature 304*:507, 1983), Nakano *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*

81:71, 1984), Taya *et al.* (EMBO J. 3:2943, 1984) and Nardeux *et al.* (*Biochem. Biophys. Res. Commun. 146*:395, 1987). The sequences of wildtype and mutant K-ras genes may also be found in Genbank under Accession Nos. K00652, K00653, K01519, K01520, K01912, L00045, L00049, M17087, M26261, M38506 and M54968.

The nucleotide sequences of wildtype and mutant N-ras genes are known (Hall *et al.*, *Nucleic Acids Res. 13*:5255, 1985; Taparowsky *et al.*, *Cell 34*:581, 1983; Geis *et al.*, *Biochem. Biophys. Res. Commun. 139*:771, 1986; Brown *et al.*, *EMBO J. 3*:1321, 1984). The sequences of wildtype and mutant N-ras genes may also be found in the Genbank and EMBOL databases under Accession Nos. K00082, L00043, M14307, X00645 and X02751.

Oligonucleotides targeted to ras genes are described in U.S. Patents Nos. 5,576,208; 5,582,972; 5,582,986; and 5,661,134, and pending application Serial No. 08/889,296, filed July 8, 1997, as well as WO 94/08003, WO 94/28720 and WO 92/22651 to Monia *et al.*, all of which are assigned to the same assignee as that of the present disclosure and which are hereby incorporated by reference.

The sequences and chemistries of oligonucleotides targeted to H-ras are detailed in Tables 1 through 7. The sequences and chemistries of oligonucleotides targeted to K-ras are detailed in Table 8. Sequences and chemistries of oligonucleotides targeted to N-ras are detailed in Table 9.

TABLE 1

Phosphorothioate Antisense Oligodeoxynucleotides

Targeted to H-ras

Targeted to the H-ras translation initiation codon

ISIS #	SEQUENCE (5'->3')	SEQ ID NO:
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

Targeted to mutant H-ras

ISIS #	TARGET	SEQUENCE (5'->3')	SEQ. ID NO:
2502	AUG	CTTATATTCCGTCATCGCTC	1
2503	AUG	TCCGTCATCGCTCCTCAGGG	2
6186	AUG	TATTCCGTCATCGCTCCTCA	7
2563	CODON 12	CGACG	8
2564	CODON 12	CCGACGG	9
2565	CODON 12	ACCGACGGC	10
2567	CODON 12	CACCGACGGCG	11
2568	CODON 12	ACACCGACGGCGC	12
2569	CODON 12	CACACCGACGGCGCC	13
3426	CODON 12	CCACACCGACGGCGCC	14
3427	CODON 12	CACACCGACGGCGCCC	15
2570	CODON 12	CCACACCGACGGCGCCC	3
3428	CODON 12	CCCACACCGACGGCGCCC	16
3429	CODON 12	CCACACCGACGGCGCCCA	17
2571	CODON 12	CCCACACCGACGGCGCCCA	4
2566	CODON 12	GCCCACACCGACGGCGCCCAC	5
2560	CODON 12	TGCCCACACCGACGGCGCCCACC	6
2561	CODON 12	TTGCCCACACCGACGGCGCCCACCA	18

2907	CODON 12 (wt)	CCACACCGCCGGCGCCC	19

TABLE 2

Chimeric Phosphorothioate Oligonucleotides Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap (Mutant Codon-12 Target)

ISIS #	# OF DEOXY RESIDUES	SEQUENCE (5'->3')	SEQ ID NO:
4122	0	**CCACACCGACGGCGCCC**	3
3975	1	**CCACACCG**A**CGGCGCCC**	3
3979	3	**CCACACC**GAC**GGCGCCC**	3
4236	4	**CCACACC**GACG**GCGCCC**	3
4242	4	**CCACAC**CGAC**GGCGCCC**	3
3980	5	**CCACAC**CGACG**GCGCCC**	3
3985	7	**CCACA**CCGACGG**CGCCC**	3
3984	9	**CCAC**ACCGACGGC**GCCC**	3
2570	17	CCACACCGACGGCGCCC	3

TABLE 3

Shortened Phosphorothioate Chimeric Oligonucleotides Derived from ISIS 3980 Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap (Mutant Codon-12 Target)

ISIS #	SEQUENCE (5'->3')	SEQ ID NO:
3980	**CCACAC**CGACG**GCGCCC**	3
4230	**CACAC**CGACG**GCGCC**	13
4276	**ACAC**CGACG**GCGC**	12
4247	**CAC**CGACG**GCG**	11
3985	**CCACA**CCGACGG**CGCCC**	3

4245	**CACA**CCGACGG**CGCC**	13
4278	**ACA**CCGACGG**CGC**	12
4229	**CA**CCGACGG**CG**	11

TABLE 4

Chimeric Phosphorothioate Oligonucleotides Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap (AUG Target)

ISIS #	# OF DEOXY RESIDUES	SEQUENCE (5'->3')	SEQ ID NO:
2502	20	CTTATATTCCGTCATCGCTC	1
4998	7	**CTTATA**TTCCGTC**ATCGCTC**	1
2503	20	TCCGTCATCGCTCCTCAGGG	2
5122	7	**TCCGTC**ATCGCTC**CTCAGGG**	2

TABLE 5

Chimeric Backbone (P=S/P=O) Oligonucleotides Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap (Backbone Linkages Indicated by "s" (P=S) or "o" (P=O) (Mutant Codon-12 Target)

ISIS #	# OF DEOXY RESIDUES	SEQUENCE (5'->3')	SEQ ID NO:
2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
4226	5	**CoCoAoCoAoCs**CsGsAsCsGo**GoCoGoCoCoC**	3
4233	11	**CsCsAsCsAsCo**CoGoAoCoGs**GsCsGsCsCsC**	3
4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4546	14	CsCsAsCsAsCsCsGoAoCsGsGsCsGsCsCsC	3
4551	13	CsCsAsCsAsCsCsGoAoCoGsGsCsGsCsCsC	3
4593	12	CsCsAsCsAsCsCoGoAoCoGsGsCsGsCsCsC	3
4606	11	CsCsAsCsAsCsCoGoAoCoGoGsCsGsCsCsC	3

4241	6	CsCsAsCoAoCoCoGoAoCoGoGoCoGsCsCsC	3

TABLE 6

Phosphorothioate Antisense Oligodeoxynucleotides Targeted to a Hairpin Structure Corresponding to Residues +18 to +64 of the Coding Sequence of Activated H-ras mRNA

ISIS #	SEQUENCE (5'->3')	SEQ ID NO:
3270	CACCACCACC	20
3271	GCGCCCACCA	21
3292	CGACGGCGCC	22
3291	CACACCGACG	23
3283	UUGCCCACAC	24
3284	CACUCUUGCC	25

TABLE 7

2'- Modified Analogs of ISIS 2503

(Positions with 2' Modifications are Emboldened)

MOE Analogs (positions with 2'-MOE are emboldened)

ISIS #	Sequence (5'->3')	SEQ ID NO:
13905	**TCCGTCA**TCGCTC**CTCAGGG**	2
13907	**TCCGTC**ATCGCTCC**TCAGGG**	2
13909	**TCCGTCA**TCGCTCCT**CAGGG**	2
13911	**TCCG**TCATCGCTCCTC**AGGG**	2
13917	TCCGTCATC**GCTCCTCAGGG**	2
13919	**TC**CGTCATCGC**TCCTCAGGG**	2
13920	**TCC**GTCATCGCT**CCTCAGGG**	2
13923	**TCCGTC**ATCGCTCCT**CAGGG**	2
13926	**TCCGTCATC**GCTCCTCAG**GG**	2
13927	**TCCGTCATCG**CTCCTCAGG**G**	2

MMI Analogs (positions with 2'-MOE are emboldened)

ISIS #	Sequence (5'->3')	SEQ ID NO:
14896	**TC**CGTCATCGCTCCTCAG**GG**	2
14897	**TC$_o$CG**TCATCGCTCCTCA**G$_o$GG**	2
14898	**TC$_s$CG**TCATCGCTCCTCA**G$_s$GG**	2
14899	**TC$_o$CG$_o$TC**ATCGCTCC**TC$_o$A$_o$GGG**	2
14900	**TC$_s$CG$_s$TC**ATCGCTCC**TC$_s$AG$_s$AG**	2

"$_o$"indicates a phosphodiester linkage between MMI dimers;
"$_s$"indicates a phosphorothioate linkage between MMI dimers.
All unmarked linkages are phosphorothioates.

TABLE 8

Phosphorothioate Antisense Oligonucleotides

Targeted to Human K-ras

Oligodeoxynucleotides

ISIS #	SEQUENCE (5'->3')	TARGET	SEQ ID NO:
6958	CTGCCTCCGCCGCCGCGGCC	5' UTR/5'-cap	28
6957	CAGTGCCTGCGCCGCGCTCG	5'-UTR	29
6956	AGGCCTCTCTCCCGCACCTG	5'-UTR	30
6953	TTCAGTCATTTTCAGCAGGC	AUG	31
6952	TTATATTCAGTCATTTTCAG	AUG	32
6951	CAAGTTTATATTCAGTCATT	AUG	33
6950	GCCTACGCCACCAGCTCCAAC	Codon 12 (wt)	34
6949	CTACGCCACCAGCTCCA	Codon 12 (wt)	35
7453	TACGCCAACAGCTCC	Codon 12 (G→T mutant)	36
6948	GTACTCCTCTTGACCTGCTGT	Codon 61 (wt)	37
6947	CCTGTAGGAATCCTCTATTGT	Codon 38	38
6946	GGTAATGCTAAAACAAATGC	3'-UTR	39
6945	GGAATACTGGCACTTCGAGG	3'-UTR	40

7679	TTTTCAGCAGGCCTCTCTCC	5'-UTR/AUG	41

Chimeric oligonucleotides having 2'-O-methyl ends (bold)

ISIS #	SEQUENCE (5'->3')	SEQ ID NO:
6957	CAGTGCCTGCGCCGCGCTCG	29
7683	**CAGTGC**CTGCGCCG**CGCTC**G	29
7679	TTTTCAGCAGGCCTCTCTCC	41
7680	**TTTTCA**GCAGGCCT**CTCTC**C	41

TABLE 9

Phosphorothioate Oligodeoxynucleotides

Targeted to Human N-ras

ISIS #	Sequence (5'->3')	Target Region	SEQ ID NO:
14677	CCGGGTCCTAGAAGCTGCAG	5' UTR	42
14678	TAAATCAGTAAAAGAAACCG	5' UTR	43
14679	GGACACAGTAACCAGGCGGC	5' UTR	44
14680	AACAGAAGCTACACCAAGGG	5' UTR	45
14681	CAGACCCATCCATTCCCGTG	5' UTR	46
14682	GCCAAGAAATCAGACCCATC	5' UTR	47
14683	AGGGGGAAGATAAAACCGCC	5' UTR	48
14684	CGCTTCCATTCTTTCGCCAT	5' UTR	49
14685	CCGCACCCAGACCCGCCCCT	5' UTR	50
14686	CAGCCCCCACCAAGGAGCGG	5' UTR	51
14687	GTCATTTCACACCAGCAAGA	AUG	52
14688	CAGTCATTTCACACCAGCAA	AUG	53
14689	CTCAGTCATTTCACACCAGC	AUG	54
14690	CGTGGGCTTGTTTTGTATCA	Coding	55
14691	CCATACAACCCTGAGTCCCA	3' UTR	56
14692	CAGACAGCCAAGTGAGGAGG	3' UTR	57

14693	CCAGGGCAGAAAAATAACAG	3' UTR	58
14694	TTTGTGCTGTGGAAGAACCC	3' UTR	59
14695	GCTATTAAATAACAATGCAC	3' UTR	60
14696	ACTGATCACAGCTATTAAAT	3' UTR	61

EXAMPLE 2: Oligonucleotide Synthesis

Substituted and unsubstituted deoxyoligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. Synthesis of 2-(amino)adenine-substituted oligonucleotides was carried out in like manner, with the following exception: at positions at which a 2-(amino)adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (Chemgenes Corp., Waltham, MA). After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides were judged from polyacrylamide gel electrophoresis to be greater than 80% full-length material.

Oligoribonucleotides were synthesized using the automated synthesizer and 5'-dimethoxy-trityl 2'-*tert*-butyldimethylsilyl 3'-O-phosphoramidites (American Bionetics, Hayward, CA). The protecting group on the exocyclic amines of A,C and G was phenoxyacetyl (Wu *et al.*, *Nucl. Acids Res.* *17*:3501, 1989). The standard synthesis cycle was modified by

increasing the wait step after the pulse delivery of tetrazole to 900 seconds. Oligonucleotides were deprotected by overnight incubation at room temperature in methanolic ammonia. After drying *in vacuo*, the 2'-silyl group was removed by overnight incubation at room temperature in 1 M tetrabutylammoniumfluoride (Aldrich Chemical Co., Milwaukee, WI) in tetrahydrofuran. Oligonucleotides were purified using a C-18 Sep-Pak cartridge (Waters Corp., Milford, MA) followed by ethanol precipitation. Analytical denaturing polyacrylamide electrophoresis demonstrated the RNA oligonucleotides were greater than 90% full length material.

EXAMPLE 3: Preparation of Sterically Stabilized Liposomes Comprising Antisense Oligonucleotides

A. Preparation of lipid film

Lipid stock solutions were prepared at 20 mg/mL in chloroform. Dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Inc., Alabaster, AL), cholesterol (Avanti Polar lipids, Inc. or Sigma Chemical Corp., St. Louis, MO) and N-(carbamoyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine-(DSPE-MPEG$_{2000}$; Avanti Polar Lipids, Inc.) were dispensed into a 30 mL round bottom flask as follows for 150 µmol of total lipid:

TABLE 10

Lipid Components of DSPE-MPEG$_{2000}$ Liposomes Comprising ISIS 2503

Component	Mole ratio	Mole %	mg lipid	mL stock lipid solution
DPPC	3	57	62.74	3.137
Cholesterol	2	38	22.03	1.102
DSPE-MPEG$_{2000}$	0.265	5	20.75	1.037

Chloroform was removed by evaporation using a rotary evaporator, heating at 60°C with a moderate vacuum. The lipid material dried as a thin film on the flask wall. Evaporation was continued using high vacuum for an additional 30 minutes at 60°C.

B. Lipid hydration

Phosphorothioate oligonucleotide (ISIS 2503) was dissolved in water to 100 mg/mL. The solution was made isotonic (80-310 mOsm) with the addition of a small quantity of 5M NaCl as needed. The final solution was filtered through a 0.22 μm membrane. Then, 0.5 mL of the resultant oligo solution was added to the flask containing the lipid film. The flask was rotated at 240 rpm at 60°C for 5 minutes. The lipid suspension was vortexed heavily to form large multi-lamellar liposomes.

The liposomes were frozen by immersing the flask into a dry ice/acetone bath for 5 minutes. Thawing of the liposomes was accomplished by immersing the flask into a 60°C water bath as necessary. The preceding freeze/thaw steps were repeated 5 times. The resulting liposome solution appeared "creamy".

C. Particle sizing

Large multi-lamellar liposomes were converted into near-uniform unilamellar liposomes by either (1) physical extrusion through polycarbonate membranes (Avestin, Inc., Ottawa, Ontario, Canada) of defined porosity (*e.g.*, 100 nm) or microfluidization with a Model 110 S microfluidizer (Microfluidics International Corp., Newton, MA). Either technique produces unilamellar liposomes of approximately 90 to about 110 nm in diameter.

D. Liposome purification

Nonencapsulated oligonucleotide material was separated from the liposomes by gel permeation chromatography using a Superdex-200 column (Pharmacia Biotech, Inc., Piscataway, NJ)

equilibrated in phosphate-buffered saline, pH 7.4. Encapsulation recovery was typically 25-30% and the final ISIS 2503 concentration in the liposomes was about 7 mg/mL. The liposome fractions were pooled and filter-sterilized through a 0.2 μm membrane (Gelman Sciences, Inc., Ann Arbor, MI). Liposomes were stored at 4°C.

EXAMPLE 4: Evaluation of Sterically Stabilized Liposomes Comprising Antisense Oligonucleotides

A. Experimental Design and Methods

Study Design: Thirteen rhesus monkeys (*Macaca mulatta*) (7 males and 6 females) were used. The animals were pre-pubertal to young adult (in the age range of 3-7 years), and their body weight ranged from 3-4 kg (Table 11). Each animal received a single intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes (10 mg/kg) over approximately 30 minutes. Blood samples for pharmacokinetic analysis were collected prior to dosing and at 0, 1, 2, 6, 12, 24, 40, 60, 96, 120, 144, 168, 192, 240, 384 and 576 hours after dosing. Animals were serial-sacrificed such that 2 animals (1 male and 1 female) were euthanized at each of the following time points from the end of infusion: 24, 60, 120, 168, 384 and 576 hours. An additional male monkey (Animal ID #R4791) died of unknown causes shortly after dosing. Although samples were analyzed for this animal, the values were not included in the pharmacokinetic analysis because the animal died before the earliest study time point. As controls in some experiments, animals were treated in the same manner but with a simple saline formulation of ISIS 2503 in saline.

TABLE 11

Animals Assigned to Study

Animal ID	Gender	Body Weight (kg)	Time Point (hr)
R4759	M	3.2	24
R3524	F	4.4	24
R4797	M	3.7	60
R2700	F	3.5	60
R4778	M	3.5	120
R4784	F	3.6	120
R4758	M	3.6	168
R4781	F	3.6	168
R4796	M	3.1	384
R4782	F	3.5	384
R4764	M	4.2	576
R4768	F	3.5	576

A full necropsy was conducted on all animals. The following tissues were collected from each animal: brain, heart, pancreas, prostate, ovaries, spleen, intestine, kidney cortex, kidney medulla, liver, mesenteric and mandibular (combined, M & M) lymph nodes, axillary and inguinal (combined, A & I) lymph nodes, lung, back skin, and hand skin. Whole blood and tissue samples were extracted and analyzed by capillary gel electrophoresis (CGE).

Sample Extraction in Whole Blood: Blood samples were vortexed and an aliquot (100 µl) was measured into a 2 mL Fastprep tube (BIO101, Inc., Vista, CA) containing approximately 1/4 inch of homogenization beads. Following the addition of 390 µL PBS, 5 µL 10% NP-40, and 5 µL 100 µM T_{27} (a 27-mer phosphorothioate oligodeoxythimidine used as the

internal standard), the mixture was homogenized in a Savant Tissue Disrupter (BIO101, Inc., Vista, CA). The samples were then extracted with phenol-chloroform to remove proteins and lipids; oligonucleotides remained in the aqueous phase. To enhance the separation of the aqueous phase from the organic phase, an aliquot of phase lock gel (Intermountain Scientific Corp., Kaysville, UT) was added to the samples after adding phenol-chloroform. The phenol-chloroform layer was back-extracted with 500 µL of water and the aqueous phases were pooled. The aqueous phase was then evaporated to dryness, re-suspended with 5 mL SAX loading buffer (containing 10 mM Tris-HCl, 0.5 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction.

Sample Extraction in Tissue: The method for tissue sample extraction combined the proteinase K digestion method previously used for extraction of oligonucleotides from tissues (Cossum *et al.*, *J. Pharmacol. Exp. Therap. 269*:89, 1994) with the solid phase extraction method (Leeds *et al.*, *Analytical Biochem. 235*:36, 1996). Monkey tissues were weighed, homogenized in a Bio Savant, and incubated for 24 hours at 37°C in a 2.0 mg/mL proteinase K solution of digestion buffer consisting of 0.5% Non-Idet P-40 (NP-40) with 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 100 mM NaCl. An appropriate amount of T_{27} ranging from 0.5 to 10 µM, was added for quantitation by capillary electrophoresis. The aqueous layer was then extracted with phenol-chloroform, the phenol-chloroform layer was back-extracted with 500 µL of water and the aqueous phases were pooled. The aqueous layer was extracted again with chloroform to remove the phenol. Samples were then evaporated to dryness, resuspended in 200 µl concentrated ammonium hydroxide and incubated at 55°C for 12 to 24 hours. The samples were then re-evaporated to dryness, resuspended with 5 mL SAX loading buffer (containing 10 mM

Tris-HCl, 0.25 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction.

Solid Phase Extraction: After phenol-chloroform extraction, both blood and tissue samples were further extracted using a J&W Scientific, Inc. (Folson, CA) strong anion exchange (SAX) SPE column. For solid phase extraction, the column was prepared for use by wetting it with 1 ml of acetonitrile followed by 1 ml of distilled water. The column was then equilibrated with 3 ml of loading buffer prior to loading the tissue or blood extracts. After loading the extracts, the anion exchange SPE column was washed with 3 mL of the loading buffer, and the oligonucleotides were eluted with 3 mL of elution buffer (containing 10 mM Tris-HCl , 0.5 M KCl, and 1.0 M NaBr, and 30% acetonitrile, at pH 9.0). The eluted samples were diluted and were then desalted using a reversed-phase solid phase extraction column.

The reversed-phase solid phase extraction column (Isolute, from Alltech Associates, Inc., Deerfield, IL) was pre-equilibrated with 1 mL acetonitrile, 1 mL distilled water, and 3 mL eluting buffer (10 mM Tris-HCl, 0.5 M KCl, and 1.0 M NaBr, at pH 9.0). After the diluted eluate from the anion exchange column was loaded onto reverse phase SPE column, it was washed with 5 mL of distilled water, and purified oligonucleotide was then eluted using 3 mL of fresh 20% acetonitrile in distilled water. After evaporation to dryness, the samples were resuspended in 40 µl distilled water, and a 15 µl aliquot was desalted by dialysis on a Millipore VS membrane (pore size 0.025 microns, Millipore Corp., Bedford, MA) floating in a 60 × 15 mm polystyrene petrie dish (Becton Dickinson and Co., Lincoln Park, NJ) containing distilled water prior to loading into microvials for analysis by capillary electrophoresis.

Capillary Electrophoresis: A Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments,

Inc., Fullerton, CA) was used for gel-filled capillary electrophoresis analysis. Samples were electrokinetically injected using an applied voltage between 3-10 kV for a duration ranging from 3-20 seconds. Length-based separation of the oligonucleotides was achieved by using a coated-capillary (Bio-Rad Laboratory, Hercules, CA) with Beckman eCAP ssDNA 100-R Gel. Separation was optimized using a constant applied voltage of 20 kV and a temperature of 40°C. Oligonucleotide peaks were detected by UV absorbance at 260 nm. Beckman System Gold Software on the P/ACE instrument was used to determine the areas under the curve for oligonucleotide peaks in the resultant electropherograms. A peak area threshold of 0.01 area units and minimum peak width of 0.08 min were the standard integration parameters (Leeds *et al.*, *Analytical Biochem. 235*:36, 1996).

Quantitation: Quantitation of intact ISIS 2503 and metabolites for whole blood samples was based on the calibration curve with T_{27} as the internal standard. The limit of quantitation for this assay has been estimated to be 0.10 µg/mL oligonucleotide in blood. In contrast, the concentrations of ISIS 2503 and metabolites in the tissue samples were calculated from the ratio of the absorbencies, based only on the starting concentration of internal standard (T_{27}) added to the samples using the following equation:

$$C_2 = C_1(E_1/E_2)[(A_2/T_{m2})/(A_1/T_{m1})]$$

Where C_1 = concentration of the internal standard, C_2= concentration of the analyte (ISIS 2503 or metabolites), E_1= molar extinction coefficient of the internal standard, E_2 = molar extinction coefficient of the analyte, A_1 = area of the internal standard peak, A_2= area of the analyte peak, T_{m1}= migration time of the internal standard peak, and T_{m2}= migration time of the analyte peak.

Calculations of extinction coefficients for ISIS 2503, metabolites, and T_{27} are made using a program which calculates

the sums of the extinction coefficients from the individual bases according to the base composition. For the calculation of extinction coefficients, metabolites are assumed, to be generated by loss of nucleotide from the 3'-end. The limit of quantitation for this assay has been estimated to be 0.10 µg/g oligonucleotide in tissue.

Pharmacokinetic Analysis: Inspection of the semi-logarithmic plots of intact ISIS 2503 (full length) blood level-versus time curves indicated that they could be described by a monoexponential equation. First order elimination was assumed. Initial estimates of parameters were obtained by linear regression of the terminal concentration time points. Nonlinear regression was accomplished using a one compartment model for each individual animal (WinNonlin 1.0, Scientific Consulting, Inc., Apex, NC). A uniform weight of 1 was used for all blood-level data. Four of the animals were excluded from complete individual pharmacokinetic analysis of blood concentrations because they were sacrificed before a complete blood profile could be collected (2 at 24 hours and 2 at 60 hours).

Tissue elimination was analyzed by noncompartmental methods using WinNonlin 1.0. Tissue half-lives were estimated by linear regression analysis of the log-linear terminal phase of the tissue concentration-time curve. The area under the tissue concentration-time curve ($AUC_{0\to\infty}$) and the area under the first moment of the concentration-time curve ($AUMC_{0\to\infty}$) were calculated using the linear trapezoidal rule, up to the last measured time point, plus the extrapolated area. The mean residence time (MRT) was calculated as the ratio of the $AUMC_{(0\to\infty)}$ to the $AUC_{(0\to\infty)}$.

Statistics: Statistical analysis for gender difference of kinetic parameters was performed by F-test (Excel 6.0, Microsoft Corp., Redmond, WA) for the analysis of variance, and t-test (Excel 6.0) for the analysis of mean at the p =

0.05 level. Descriptive statistics were used to present data summaries for pharmacokinetic parameter estimates and blood concentration data.

B. Results

Blood Pharmacokinetics and Metabolism: Figures 6 and 7 are representative electropherograms of (a) liposomal and (b) saline formulations of ISIS 2503 in blood and kidney samples, respectively, from monkeys after i.v. infusions of 10 mg/kg of the respective formulations. The saline formulated oligonucleotide samples were taken either 1 hour after initiation of a 2-hour infusion in the case of plasma or 48 hours after the last 2-hour infusion of 14 doses administered every other day (q2d). In contrast, the liposomal oligonucleotide formulations were evaluated at 60 hours after an 0.5 hour infusion. Despite the longer period during which the liposomal oligonucleotide formulations were exposed to degradative processes in the tissues, the ISIS 2503 remained in a significantly more intact state than the saline-formulated oligonucleotide (as can be seen by comparing panel (a) in Figures 6 and 7 to panel (b)).

The time course of the clearance of ISIS 2503 and oligonucleotide metabolites from blood after administration of the liposomal oligonucleotide composition is prolonged (see Tables 12 and 13 and Figure 1). Maximum blood concentration (C_{max}) of intact ISIS 2503 was approximately 90 µg/mL and was observed at the end of the 30-minute infusion. ISIS 2503 concentration did not decrease by 1 hour after infusion but remained at c. 90 µg/mL). Concentrations in blood decreased slowly to approximately 10 µg/mL at 144 hours after infusion. In these experiments, the method for quantitating ISIS 2503 concentrations in blood or tissues does not distinguish between free and liposome encapsulated oligonucleotides, and both parent compound and total oligonucleotide concentrations are presented because many of the chain-shortened metabolites

retain physical and chemical properties similar to those of the parent compound ISIS 2503 and thus may potentially have some biological activity.

Pharmacokinetic parameter estimates for males and females were averaged since statistical analysis indicated no significant gender differences. The mean blood half-life for intact ISIS 2503 was 57.2 hours (Table 14). The concentration of ISIS 2503 in blood generally fell below the limit of detection after 168 hours. The observed concentrations in blood were less well predicted by the model after 120 hours (Figure 1). The values predicted by the model were higher than the actual values observed at the late time points suggesting that there were alterations in the kinetics after extended circulation times. This phenomenon may be a result of the loss of liposome integrity after prolonged circulation in blood. The average total body clearance and Vd_{ss} were 1.53 ± 0.28 mL/hr/kg and 123 ± 28 mL/kg, respectively. The volume of distribution was larger than the blood compartment (73.4 mL/kg) indicating some distribution into tissues, but also indicated a large portion of administered dose remained in the general circulation (Davies *et al.*, *Pharm. Res. 10*:1093, 1993).

The metabolites of ISIS 2503 in blood co-migrated on CGE with ISIS 2503 shortened by removal of 1 or 2 bases (19-mer and 18-mer; in Tables 12, 13 and 15 these are referred to as "n-1" and "n-2," respectively). Concentrations of metabolites observed were an order of magnitude lower than that of parent drug. The chain-shortened metabolites cumulatively represented approximately 5% to 20% of the total oligonucleotides in blood. There was only a small increase in the percentage of oligonucleotide metabolites with time. This pattern of very low concentrations of metabolites observed in blood suggests that liposomal encapsulation protected the oligonucleotide from blood (and tissue)

nucleases that might otherwise rapidly metabolize the circulating oligonucleotide, and supports the notion that there was very little leakage of ISIS 2503 from the liposome.

TABLE 12

Concentrations (μg/mL) of ISIS 2503 and All Detected Metabolites in Blood After 0.5 hr Intravenous Infusion of 10 mg/kg ISIS 2503 Encapsulated in Sterically Stabilized Liposomes to Rhesus Monkeys

Time (hr)	No. of Animals	Mean Concentration (μg/mL) ISIS 2503	n-1	n-2	Total	% Full Length
0	12	89.0[a] (24.7)[b]	4.27 (3.54)	nd	93.3 (25.4)	95.5 (3.5)
1	12	90.4 (18.9)	3.44 (2.24)	0.28 (0.96)	94.1 (20.0)	96.1 (2.5)
2	12	82.1 (22.0)	3.27 (2.10)	nd	85.4 (22.2)	96.1 (2.6)
6	12	78.3 (20.4)	2.92 (1.64)	0.31 (1.06)	81.5 (20.6)	96.0 (2.6)
12	12	64.6 (18.7)	1.35 (1.58)	0.28 (0.96)	66.2 (19.7)	97.8 (2.9)
24	12	63.3 (18.2)	2.08 (1.26)	nd	65.4 (18.3)	96.7 (2.2)
40	10	50.1 (15.0)	1.45 (1.02)	0.06 (0.17)	51.9 (15.4)	96.9 (1.8)
60	10	50.6 (9.5)	1.37 (1.02)	0.29 (0.91)	56.7 (16.6)	92.2 (14.0)
96	8	22.6 (12.9)	2.65 (2.23)	nd	25.3 (14.6)	91.9 (7.2)
		15.7	2.61		18.3	89.2

120	7	(10.2)	(1.91)	nd	(12.0)	(8.0)
144	4	10.6 (6.3)	1.07 (2.14)	nd	11.7 (7.4)	94.2 (11.6)
168	4	4.42 (1.32)	0.40 (0.69)	nd	4.82 (1.67)	93.5 (11.3)
192	2	3.62	0.59 (0.83)	nd	4.21 (0.60)	84.4 (22.0)
240	1	2.13	1.19	nd	3.32	64.2

"% Full-length" = percent of total detectable oligonucleotide represented by intact ISIS 2503.

"nd" = not detected (detection level = 0.10 µg/mL).

[a] Mean value.

[b] Standard deviation.

TABLE 13

ISIS 2503 and Total Oligonucleotide Whole Blood Concentrations after 0.5 hr i.v. Infusion of 10 mg/kg ISIS 2503 Encapsulated in Sterically Stabilized Liposomes to Rhesus Monkeys (Average of Duplicate Analysis)

Animal ID #	Gender	Time[a] (hr)	µg/mL ISIS 2503	n-1	n-2	n-3	Total	% Full[b]
R4759	M	0	115	1.06	nd	nd	116	99.1
R4759	M	1	120	6.58	3.32	nd	130	92.4
R4759	M	2	93.8	6.11	nd	nd	99.9	93.9
R4759	M	6	89.8	5.71	3.66	nd	99.2	90.6

R4759	M	12	78.7	5.30	3.33	nd	87.3	90.1
R4759	M	24	58.9	4.09	nd	nd	63.0	93.5
R3524	F	0	131	1.11	nd	nd	132	99.2
R3524	F	1	92.2	0.33	nd	nd	92.5	99.6
R3524	F	2	92.9	0.58	nd	nd	93.5	99.4
R3524	F	6	102	0.90	nd	nd	103	99.1
R3524	F	12	98.8	0.77	nd	nd	99.6	99.2
R3524	F	24	76.4	0.11	nd	nd	76.5	99.9
R4797	M	0	84.5	6.33	nd	nd	90.8	93.0
R4797	M	1	85.1	6.42	nd	nd	91.5	93.0
R4797	M	2	71.4	6.30	nd	nd	77.7	91.9
R4797	M	6	66.1	3.65	nd	nd	69.8	94.8
R4797	M	12	68.5	nd	nd	nd	68.5	100
R4797	M	24	53.8	3.17	nd	nd	56.9	94.4
R4797	M	40	40.4	0.89	nd	nd	41.3	97.8
R4797	M	60	51.4	0.49	nd	nd	96.3	53.4
R2700	F	0	75.7	6.61	nd	nd	82.3	92.0
R2700	F	1	82.1	6.62	nd	nd	88.7	92.5
R2700	F	2	64.9	6.15	nd	nd	71.1	91.3
R2700	F	6	68.8	6.03	nd	nd	74.8	91.9
R2700	F	12	45.1	nd	nd	nd	45.1	100

	R2700	F	24	58.7	1.48	nd	nd	60.2	97.5
	R2700	F	40	44.4	1.34	0.55	nd	46.3	95.9
	R2700	F	60	61.3	0.72	nd	nd	62.0	98.8
	R4778	M	0	96.3	10.3	nd	nd	107	90.3
	R4778	M	1	124	1.58	nd	nd	126	98.7
	R4778	M	2	133	2.59	nd	nd	135	98.1
	R4778	M	6	128	2.78	nd	nd	131	97.9
	R4778	M	12	94.7	2.60	nd	nd	97.3	97.3
	R4778	M	24	106	2.05	nd	nd	108	98.1
	R4778	M	40	44.6	1.52	nd	nd	46.1	96.7
	R4778	M	60	42.3	1.03	nd	nd	43.4	97.6
	R4778	M	96	5.03	nd	nd	nd	5.03	100
	R4784	F	0	92.9	10.5	nd	nd	103	89.8
	R4784	F	1	89.6	1.94	nd	nd	91.5	97.9
	R4784	F	2	95.0	1.96	nd	nd	96.9	98.0
	R4784	F	6	85.0	1.34	nd	nd	86.3	98.5
	R4784	F	12	45.7	nd	nd	nd	45.7	100
	R4784	F	24	70.9	2.05	nd	nd	72.9	97.2
	R4784	F	40	48.3	nd	nd	nd	91.5	97.9
	R4784	F	60	53.4	0.97	nd	nd	54.3	98.2
	R4784	F	96	43.5	3.99	nd	nd	47.4	91.6

R4784	F	120	23.3	3.45	nd	nd	26.8	87.1
R4758	M	0	109	4.91	nd	nd	114	95.7
R4758	M	1	78.1	3.08	nd	nd	81.2	96.2
R4758	M	2	69.0	2.60	nd	nd	71.6	96.4
R4758	M	6	75.1	2.99	nd	nd	78.1	96.2
R4758	M	12	62.4	0.87	nd	nd	63.3	98.6
R4758	M	24	85.6	3.35	nd	nd	89.0	96.2
R4758	M	40	48.1	1.22	nd	nd	49.3	97.5
R4758	M	60	52.1	0.71	nd	nd	52.8	98.7
R4758	M	96	32.2	4.78	nd	nd	37.0	87.1
R4758	M	120	18.3	2.48	nd	nd	20.8	88.1
R4758	M	144	17.3	nd	nd	nd	17.3	100
R4758	M	168	11.8	nd	nd	nd	11.8	100
R4781	F	0	90.6	4.07	nd	nd	94.6	95.7
R4781	F	1	96.1	4.24	nd	nd	100	95.8
R4781	F	2	105	5.26	nd	nd	110	95.2
R4781	F	6	67.1	3.12	nd	nd	70.2	95.6
R4781	F	12	43.9	nd	nd	nd	43.9	100
R4781	F	24	53.4	2.66	nd	nd	56.0	95.3
R4781	F	40	51.8	1.57	nd	nd	53.4	97.1
R4781	F	60	52.9	0.85	nd	nd	53.8	98.4

R4781	F	96	26.0	4.48	nd	nd	30.5	85.3
R4781	F	120	15.5	4.28	nd	nd	19.7	78.3
R4781	F	144	3.46	nd	nd	nd	3.46	100
R4781	F	168	2.93	nd	nd	nd	2.93	100
R4782	M	0	30.9	0.30	nd	nd	31.2	99.1
R4782	M	1	72.4	1.42	nd	nd	73.8	98.1
R4782	M	2	60.4	1.16	nd	nd	61.5	98.1
R4782	M	6	62.1	1.32	nd	nd	63.4	97.9
R4782	M	12	58.3	1.05	nd	nd	59.4	98.2
R4782	M	24	51.8	0.89	nd	nd	52.7	98.3
R4782	M	40	37.5	2.56	nd	nd	40.1	93.6
R4782	M	60	43.8	3.84	2.89	nd	50.5	86.7
R4782	M	96	26.8	3.50	nd	nd	30.3	88.5
R4782	M	120	19.1	3.52	nd	nd	22.6	84.4
R4782	M	144	14.1	4.27	nd	nd	18.4	76.8
R4782	M	168	5.45	nd	nd	nd	5.45	100
R4782	M	192	4.64	nd	nd	nd	4.64	100
R4796	F	0	76.1	2.70	nd	nd	78.8	96.6
R4796	F	1	106	4.74	nd	nd	111	95.7
R4796	F	2	64.1	2.36	nd	nd	66.4	96.5
R4796	F	6	73.4	3.23	nd	nd	76.6	95.8

R4796	F	12	63.0	1.40	nd	nd	64.4	97.8
R4796	F	24	49.1	0.89	nd	nd	50.0	98.2
R4796	F	40	43.1	2.13	nd	nd	45.2	95.3
R4796	F	60	43.7	1.21	nd	nd	44.9	97.3
R4796	F	96	21.1	4.44	nd	nd	25.5	82.6
R4796	F	120	29.3	4.58	nd	nd	33.8	86.5
R4796	F	144	7.42	nd	nd	nd	7.42	100
R4796	F	168	4.89	1.19	nd	nd	6.08	80.4
R4796	F	192	2.61	1.18	nd	nd	3.79	68.9
R4796	F	240	2.13	1.19	nd	nd	3.32	64.2
R4768	F	0	80.5	1.68	nd	nd	82.1	98.0
R4768	F	1	76.4	1.35	nd	nd	77.8	98.3
R4768	F	2	72.2	1.30	nd	nd	73.5	98.2
R4768	F	6	69.6	1.46	nd	nd	71.0	97.9
R4768	F	12	69.4	1.31	nd	nd	70.7	98.1
R4768	F	24	50.4	0.84	nd	nd	51.3	98.4
R4768	F	40	90.6	3.25	nd	nd	93.8	96.5
R4768	F	60	68.7	2.44	nd	nd	71.1	96.6
R4768	F	96	5.61	nd	nd	nd	5.61	100
R4768	F	120	3.24	nd	nd	nd	3.24	100
R4764	M	0	85.7	1.64	nd	nd	87.4	98.1
R4764	M	1	61.8	2.99	nd	nd	64.8	95.4

R4764	M	2	63.9	2.89	nd	nd	66.8	95.7
R4764	M	6	52.8	2.57	nd	nd	55.4	95.4
R4764	M	12	46.7	2.88	nd	nd	49.6	94.2
R4764	M	24	44.5	3.35	nd	nd	47.8	93.0
R4764	M	40	52.0	nd	nd	nd	52.0	100
R4764	M	60	37.0	1.40	nd	nd	38.4	96.4
R4764	M	96	20.8	nd	nd	nd	20.8	100
R4764	M	120	1.16	nd	nd	nd	1.16	100

"nd" = not detected

[a] Time is given in hours.

[b] "%Full" = % full-length oligonucleotide detected.

TABLE 14

Summary of Estimated Pharmacokinetic Parameters (n=8) for ISIS 2503 (10 mg/kg) Encapsulated in Sterically Stabilized Liposomes Administered to Rhesus Monkeys by 0.5 hr Infusion

Parameter	**Mean**	**SD**	**CV %[b]**
AUC (µg•hr/mL)	6760	1240	18.4
K_{10}-$t_{1/2}$ (hr)	57.2	14.2	24.9
Cmax (µg/mL)[a]	90.4	23.0	26.9
Cl (mL/hr/kg)	1.52	0.28	18.3
MRT (hr)	82.5	20.5	24.9
Vd_{ss} (mL/kg)	123	28	22.3

[a] Data obtained from 12 animals.

[b] CV% = Coefficient of Variation = (Standard deviation / Mean) x 100.

In sum, encapsulation of phosphorothioate oligonucleotide into liposomes greatly modified oligonucleotide pharmacokinetics. ISIS 2503 in liposomes was cleared slowly from the blood compared with previous experience with unencapsulated oligonucleotide. Phosphorothioate oligonucleotide concentration following intravenous infusion of unencapsulated oligonucleotide in monkeys decreases rapidly from circulation with an average distribution half- life of 36-83 minutes (Agrawal *et al.*, *Clinical Pharmacokinet. 28*:7, 1995). In contrast, the distribution phase half-life of ISIS 2503 in this liposome formulation was markedly longer (approximately 57 hours), and resulted in an AUC that was approximately 70-fold greater than an equivalent dose of an unencapsulated oligonucleotide.

Tissue Distribution, Elimination Kinetics and Metabolism: ISIS 2503 was distributed widely into all the tissues analyzed. The highest tissue concentrations of total oligonucleotide were measured in liver, with slightly lower concentrations detected in spleen, followed by the lymph nodes, lung, hand skin, kidney cortex and medulla, heart, back skin, pancreas, colon, and brain (Table 15, Figures 2-5). It appears that the primary organs of ISIS 2503 distribution were the organs of the reticulo-endothelial system. Largest sample to sample variability was observed in skin where, presumably, the thickness of the skin layer collected varied greatly. Tissue distribution was also greatly different for the liposome formulation compared with unencapsulated oligonucleotides studied previously (Agrawal *et al.*, *Clinical Pharmacokinet. 28*:7, 1995; Cossum *et al.*, *J. Pharmacol. Exp. Therap. 267*:1181, 1993), where the highest concentration of oligonucleotide is consistently observed in kidney.

Relatively long half-lives of ISIS 2503 were observed in all tissues studied (Table 16). The mean residence time (15 days) of ISIS 2503 in the kidney cortex was the longest among all the tissues examined. This slow clearance may represent slow metabolism in the kidney or, alternatively, the kidney may take up free oligonucleotide from the circulation as it is slowly released from liposomes, thus giving the appearance of prolonged half-life. Uptake was slow in all tissues with a time to peak concentration from 1 to 7 days. The concentration of ISIS 2503 in brain, prostate, and ovaries was still increasing up to seven days after dosing. However, the concentration of ISIS 2503 in these tissues was below the limit of quantitation for the CGE analysis by 384 hours (the next data point after the 7-day time point).

TABLE 15

Average (n=2) Tissue Concentrations (μg/g) of ISIS 2503 and All Detected Metabolites After 0.5 hr Intravenous Infusion of 10 mg/kg ISIS 2503 Encapsulated in Sterically Stabilized Liposomes to Rhesus Monkeys

Time (hr)	(μg/mL) 2503	n+1	n-1	n-2	n-3	n-4	n-5	n-6	Total	% Full Length
Kidney Cortex										
24	0.37	13.4	0.73	0.38	0.46	0.24	0.11	0.07	15.7	77.2
60	0.24	11.2	0.61	0.34	0.13	0.09	0.05	0.04	12.7	88.7
120	0.12	4.87	0.44	0.38	0.38	0.17	0.20	0.06	6.66	72.1
168	0.18	6.00	0.72	0.22	0.94	0.48	0.36	0.16	9.31	66.2
384	0.13	1.97	0.36	0.43	0.52	0.39	0.22	0.10	4.35	45.3
576	0.07	0.90	0.19	0.20	0.09	nd	nd	nd	1.45	62.1

Kidney Medulla										
24	0.47	14.3	0.33	0.08	0.17	0.18	0.07	nd	15.6	92.6
60	0.16	13.3	0.51	0.15	0.28	0.11	0.05	0.09	14.8	91.3
120	0.18	7.48	0.53	0.39	0.40	0.28	0.29	0.11	10.1	73.2
168	0.38	7.17	0.69	0.39	0.54	0.38	0.23	0.14	10.6	67.8
384	0.06	2.40	0.33	0.41	0.17	0.12	0.21	0.21	4.05	59.3
576	0.05	0.93	0.32	0.26	0.24	0.06	0.05	0.06	1.99	46.7
Liver										
24	0.87	46.7	2.22	0.65	0.99	0.44	0.15	0.03	52.1	90.0
60	0.22	94.1	4.60	1.78	2.73	0.93	0.59	0.47	119	80.0
120	0.50	88.9	7.17	3.22	4.55	1.71	1.13	1.09	110	80.9
168	0.91	106	10.2	4.62	7.59	2.76	1.94	1.64	140	72.8
384	0.28	13.45	3.27	1.76	3.35	1.51	1.15	1.12	28.9	46.5
576	0.17	7.49	1.96	0.84	1.94	0.81	0.39	0.20	15.3	49.0
Spleen										
24	0.51	62.0	2.92	0.93	0.87	0.15	nd	nd	67.4	92.0
60	0.60	84.4	4.17	1.85	2.19	1.39	0.62	0.51	96.9	87.0
120	0.26	90.0	5.98	2.63	3.65	1.61	0.94	1.62	106	83.8
168	0.87	94.0	6.20	2.54	3.24	1.15	0.63	0.39	109	85.9
384	0.12	26.6	2.75	1.48	2.40	1.40	1.26	1.20	43.1	61.6
576	0.08	26.6	1.78	0.71	1.18	0.56	0.42	0.51	32.7	81.3
Back Skin										
24	0.16	3.63	nd	nd	nd	nd	nd	nd	3.79	94.5

60	0.04	2.80	0.07	0.03	0.03	nd	nd	nd	2.96	95.0
120	0.03	4.63	0.16	0.06	0.06	0.02	0.01	nd	4.96	93.3
168	0.08	11.1	0.53	0.18	0.19	0.06	0.04	0.01	12.2	93.2
384	0.02	0.24	0.02	nd	nd	nd	nd	nd	0.26	88.7
576	0.01	0.09	nd	nd	nd	nd	nd	nd	0.10	89.5
Hand Skin										
24	0.14	13.6	0.23	0.03	0.04	0.02	0.02	0.01	14.1	96.4
60	0.13	23.9	0.68	0.20	0.11	0.09	0.06	0.03	25.2	94.2
120	0.17	24.6	0.92	0.22	0.29	0.08	0.04	0.03	26.4	93.3
168	0.32	25.7	1.02	0.27	0.39	0.17	0.21	0.07	28.3	92.2
384	0.13	13.54	1.11	1.46	0.47	0.11	0.05	0.04	17.02	79.6
576	0.10	1.10	0.13	0.06	0.16	0.03	nd	nd	1.56	70.5
A & I Lymph Nodes										
24	0.26	27.7	0.85	0.26	0.17	0.02	nd	nd	29.3	94.6
60	0.05	43.5	2.07	0.57	0.96	0.38	0.13	nd	47.6	91.2
120	0.24	68.2	4.16	1.61	3.10	1.08	0.23	0.07	78.7	86.6
168	1.03	42.4	4.05	2.03	2.99	1.30	0.41	0.36	55.4	77.0
384	0.26	29.7	2.33	0.99	1.85	0.61	0.61	0.46	39.2	75.8
576	0.23	14.6	0.79	0.28	0.55	0.26	0.26	0.14	18.0	81.1
M & M Lymph Nodes										
24	0.21	9.47	0.49	0.37	0.11	0.11	0.07	0.05	11.0	86.0

60	0.23	14.4	1.03	0.82	0.50	0.29	0.22	0.16	17.8	81.1
120	0.22	43.8	3.18	1.38	2.19	0.65	0.31	0.18	52.0	83.9
168	0.18	38.3	3.80	1.97	2.61	1.02	0.56	0.43	49.1	79.1
384	0.10	15.1	1.28	0.58	1.08	0.44	0.38	0.47	21.8	69.3
576	0.05	8.01	0.37	0.18	0.20	0.09	0.05	0.07	9.24	88.9

Brain

24	0.06	2.21	0.03	nd	nd	nd	nd	nd	2.30	96.0
60	0.06	1.28	0.01	nd	nd	nd	nd	nd	1.35	92.5
20	0.06	0.94	nd	nd	nd	nd	nd	nd	1.00	94.2
168	0.05	2.27	nd	nd	nd	nd	nd	nd	2.32	97.6

Colon

24	0.05	5.44	0.13	0.05	0.01	0.01	0.01	0.01	5.71	95.2
60	0.14	4.02	0.19	0.11	0.09	0.03	0.02	nd	4.65	86.3
120	0.03	6.88	0.65	0.35	0.39	0.05	0.01	nd	8.35	82.3
168	0.08	6.48	0.47	0.55	0.06	0.05	0.04	0.01	7.75	86.8

Heart

24	0.04	10.3	0.28	nd	nd	nd	nd	nd	10.6	97.2
60	0.12	6.28	0.20	0.06	0.06	nd	nd	nd	6.72	94.2
120	0.05	3.72	0.11	nd	nd	nd	nd	nd	3.89	95.8
168	0.07	2.78	0.10	0.05	nd	nd	nd	nd	2.99	91.3

Lung

24	0.14	22.5	0.44	0.20	0.03	nd	nd	nd	23.3	96.6

60	0.05	26.1	0.22	0.02	nd	nd	nd	nd	26.4	98.5
120	0.10	6.95	0.22	nd	nd	nd	nd	nd	7.28	93.8
168	0.19	4.45	0.09	0.03	0.02	0.01	nd	nd	4.79	90.5

Pancreas

24	0.11	3.63	0.14	0.20	0.35	0.36	0.72	0.60	9.41	41.2
60	0.12	5.53	0.30	0.13	0.30	0.19	0.44	0.63	10.6	54.6
120	0.09	2.62	0.16	0.12	0.27	0.35	0.24	0.46	4.75	53.8
168	0.07	1.78	0.25	0.33	0.09	0.06	0.30	0.55	3.46	53.2

Prostate

24	0.11	3.02	0.01	0.03	nd	nd	nd	nd	3.13	96.5
60	0.12	3.72	0.17	0.13	nd	nd	nd	nd	4.28	81.5
120	0.09	2.35	0.16	0.09	0.42	0.19	0.26	0.11	3.75	64.7
168	0.07	6.89	0.49	0.21	0.36	0.12	0.10	0.14	8.63	80.1

Ovary

24	0.09	5.18	0.20	0.16	0.13	nd	nd	nd	5.77	89.8
60	0.52	6.93	0.29	0.17	0.11	0.06	0.01	0.03	8.15	85.1
120	0.13	5.49	0.29	0.22	0.15	1.97	0.08	0.05	8.63	65.2
168	0.26	6.58	0.91	0.41	0.79	0.30	0.26	0.43	10.5	62.7

nd" = not detected (detection level = 0.10 µg/mL)

TABLE 16

Estimated Tissue Pharmacokinetic Parameters for ISIS 2503 (10 mg/kg) Encapsulated in Sterically Stabilized Liposomes Administered to Rhesus Monkeys by 0.5 hr Intravenous Infusion

Tissue	$T_{1/2}$ (day)	MRT (day)	T_{max} (day)	C_{max} (µg/g)
Kidney Cortex	11	15	1	23.3
Kidney Medulla	5.6	8.2	1	22.6
Liver	4.2	8.1	7	160
A & I Lymph Node	NA[a]	18	5	97.0
M & M Lymph Node	7.7	13	5	57.4
Spleen	9.7	14	5	107
Back Skin	3.1	7.0	7	16.5
Hand Skin	4.2	10	2	43.8
Lung	2.0	3.4	2	32.3
Heart	3.0	5.7	1	12.7
Pancreas	3.2	6.4	2	9.43
Brain	NA	NA	1	2.41
Colon	NA	NA	7[b]	8.24
Ovary	NA	NA	2	6.92
Prostate	NA	NA	7[b]	6.89

[a] "NA" = not available

[b] Concentration was still increasing at the last analyzed time point.

The appearance of metabolites was low even 576 hours after infusion (Table 15). Very low relative percentage of

metabolites were observed for all the organs (~10-20%) except for the liver, the kidney cortex, and the pancreas (~30-60%). Higher concentration of oligonucleotide metabolites was observed as early as 24 hours after infusion in the pancreas. Although not wishing to be bound by any particular theory, this phenomenon could be related to the activity of lipases in this organ allowing more ISIS 2503 to escape from liposomes and be metabolized (McNeely *et al.*, "Pancreas Function" In: *Clinical Chemistry: Theory, Analysis, and Correlation*, Kaplan and Pesce, eds., The C.V. Mosby Company, St. Louis, pp. 390-397, 1989). At later time points (≥ 120 hr), increasing concentrations of chain-shortened oligonucleotide metabolites were seen in liver and kidney. Kidney and/or liver may also play a role in the degradation of liposomes but, alternatively, may be primary sites of free oligonucleotide and metabolite distribution.

In addition to chain-shortened metabolites, there were also UV absorbing peaks that migrated more slowly than parent oligonucleotide. Slower migrating oligonucleotide peaks have been identified for other phosphorothioate oligonucleotides in tissue. Slower migration suggests that the mass to charge ratio was increased either from the addition of a substituent or loss of charge. These metabolites are thought to represent intact drugs plus an additional substituent possibly an additional nucleotide or two (Griffey *et al.*, *J. Mass. Spec.* *32*:305, 1997). Thus, while not wishing to be bound by any particular theory, it is possible that the slower migrating peak observed in these studies is such a lengthened metabolite, and this peak is thus referred to as "n+1" in Table 15.

Toxicokinetic Summary and Conclusions: In this investigation, it has been demonstrated that ISIS 2503 in a sterically stabilized liposome formulation has a markedly prolonged circulation time. Maximum concentration (C_{max}) in blood is achieved at the end of infusion and it is

approximately 90 μg/mL. Pharmacokinetic modeling of ISIS 2503 indicates a slow distribution process with a half-life of approximately 57 hours. The half-life of ISIS 2503 in this formulation is significantly greater than that observed for unencapsulated oligonucleotides suggesting that ISIS 2503 in liposomes is slowly distributed to tissues and protected from metabolism in blood. Unencapsulated oligonucleotide is cleared from plasma by a combination of metabolism and tissue distribution. Unencapsulated oligonucleotide has been reported to have half-lives ranging from 36-83 minutes. With this formulation there appears to be little metabolism, and clearance from blood is slow with a half-life of 57 hours. Clearly this formulation has altered the kinetics of circulating oligonucleotide. While not wishing to be bound by any particular theory, because tissue distribution is the primary route for both liposomal oligonucleotide and unencapsulated oligonucleotide clearance from circulation, slower kinetics of liposome uptake seen in tissue may explain the prolonged circulation of oligonucleotide in this study.

Liposomal ISIS 2503 is widely distributed into all tissues tested, in descending maximum concentration (C_{max}) order, liver> spleen> lymph nodes> hand skin> lung> kidney> back skin> heart> pancreas> colon> ovary> prostate> brain. Intact ISIS 2503 is the predominant oligonucleotide species measured indicating a slow metabolism in tissues and supporting the concept that liposomes remain intact in tissues. The apparent increase in metabolites observed in kidney, liver, and pancreas could be explained by digestion of the liposomes in these tissues, or preferential uptake of metabolites from circulation by these tissues. The high oligonucleotide concentrations in liver and spleen suggest that liposome formulations are primarily removed from blood by the reticulo-endothelial system. The persistence and abundance of intact ISIS 2503 in tissues is best explained

by the protection from nucleases afforded by liposomal encapsulation.

EXAMPLE 5: Evaluation of the Antitumor Activity of Sterically Stabilized Liposomes Comprising Antisense Oligonucleotides

One advantage of some sterically stabilized liposomes is their ability to deliver conventional chemotherapeutic agents to tissues, particularly tumors, other than those of the reticuloendothelial system (RES) (Gabizon *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 85*:6949, 1988; Papahadjopoulos *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 88*:11460, 1991). In disease states where leaky vasculature is characteristic (*e.g.*, inflammation, tumors), prolonging the circulation time via the liposomal oligonucleotide formulations of the invention may allow for more effective delivery of oligonucleotide as well as providing for a less frequent oligonucleotide dosing interval. In order to test the efficacy of liposomal oligonucleotide formulations against tumors, a human-mouse xenograft model was used.

A. Experimental Design and Methods

Liposomes: Sterically stabilized liposomes comprising DSPE-MPEG$_{2000}$ in the lipid phase and ISIS 2503 in the aqueous phase were prepared as in Example 3. ISIS 2503 loaded sterically stabilized liposomes comprising the monosialoganglioside G_{M1} instead of DSPE-MPEG$_{2000}$ were prepared in like fashion, except that monosialoganglioside G_{M1} (Sigma Chemical Co., St. Louis, MO) was substituted for DSPE-MPEG$_{2000}$ at the same final molar concentration. In some experiments, ISIS 13177 was used as a control. This phosphorothioate oligodeoxynucleotide has the nucleotide sequence 5'-TCAGTAATAGCCCCACATGG (SEQ ID NO: 26). In other experiments, ISIS 2105 was used as a control. This oligonucleotide has the nucleotide sequence 5'-TTGCTTCCATCTTCCTCGTC (SEQ ID NO: 27), which is targeted to the E2 gene of papillomavirus HPV-11.

Saline formulations of ISIS 2503 were also included as controls in the experiments.

Xenografts: Xenografts of human tumor cell lines into BALB/c nude mice were performed essentially as described by Dean *et al.* (*Cancer Res.* *56*:3499, 1996). Cell lines NCI-H69 and MIA PaCa-2 are available from the American Type Culture Collection (A.T.C.C., Rockville, MD) as accession numbers ATCC HTB-119 and ATCC CRL-1420, respectively.

Dosing and Analysis: Formulations were administered intraperitoneally (i.p.) or intravenously (i.v.) at the indicated frequencies including every other day (q2d) and every third day (q3d). Tumor volume was measured at the indicated times by measuring perpendicular diameters and calculated as described Dean *et al.*, *Cancer Res.* *56*:3499, 1996). For distribution studies, mice were given two doses of 10 mg/kg of formulation and then sacrificed after 24 hours. Tumor tissue was removed and analyzed by capillary electrophoresis for the presence of various oligonucleotide species as described in Example 4.

B. Results

Distribution: Sterically stabilized liposomes comprising either DSPE-MPEG$_{2000}$ (PEG) or monosialoganglioside G_{M1} (GM1) resulted in enhanced delivery of ISIS 2503 to H69 and Mia PaCa tumor cells (Tables 17 and 18, respectively). The enhanced delivery was observed both in terms of increased concentration and total amount of oligonucleotide delivered to tumor tissue, and as a percentage of the total dose of oligonucleotide administered to each animal. Of particular significance is the fact that significant improvements in the percentage of intact oligonucleotide delivered to the tumor tissue increased from less than about 4% (saline formulation) to about 11% (liposomes with G_{M1}) to over 15% (liposomes with PEG) (Table 18).

TABLE 17

Distribution of ISIS 2503 in Tumors in Mice with H69 Xenografts 24 Hours After Two Doses of 10 mg/kg

Formulation	n[a]	Conc. (ug/g) Avg.[b]	Conc. (ug/g) SD[c]	Amount (ug) Avg.	Amount (ug) SD	% of Dose Avg.	% of Dose SD
Saline	3	3.51	0.47	1.35	0.40	0.29	0.11
Liposomes-PEG	2	17.82	7.37	4.82	3.61	1.11	0.91
Liposomes-GM1	2	10.01	3.24	5.40	3.85	3.08	2.08

[a] "n" = number of animals.

[b] "Avg." = average (mean).

[c] "SD" = standard deviation.

TABLE 18

Distribution of ISIS 2503 in Tumors in Mice with MIA PaCa Xenografts 24 Hours After Two Doses of 10 mg/kg

Formulation	n	Conc. (µg/g) Avg.	Conc. (µg/g) SD	Amount (µg) Avg.	Amount (µg) SD	% of Dose Avg.	% of Dose SD
A. Total concentration of ISIS 2503 & metabolites							
Saline	3	3.82	1.96	3.74	2.32	0.09	0.06
Liposomes-PEG	3	16.21	4.98	15.27	10.93	0.36	0.25
Liposomes-GM1	3	15.80	8.99	10.69	2.96	0.26	0.06
B. Concentration of full-length ISIS 2503							
Saline	3	0.87	0.12	0.09	0.06	3.74	3.74
Liposomes-PEG	3	9.95	2.75	0.36	0.25	15.27	15.27
Liposomes-GM1	3	8.56	5.18	0.26	0.06	10.69	10.69

Antitumor Activity: The liposomal oligonucleotide formulations of the invention were evaluated for their ability to control the growth of human tumor cells transplanted into BALB/c nude mice. One such experiment, in which liposomes comprising ISIS 2105 were used as a control formulation, is shown in Table 19.

TABLE 19

Antitumor Activity of Liposomal Formulations of ISIS 2503 Against MIA PaCa Xenografts

Formulation:	Day	n	Tumor Size (mm^3) Mean	SD	Std. Error
Saline/no oligonucleotide					
	10	8	0.115	0.036	0.013
	14	8	0.321	0.119	0.042
	21	8	0.964	0.417	0.148
	30	8	1.544	0.708	0.250
PEG-Liposome/ISIS 2503 (1 mg/kg)					
	10	7	0.116	0.033	0.012
	14	7	0.216	0.101	0.038
	21	7	0.700	0.335	0.127
	30	7	1.480	0.851	0.322
PEG-Liposome/ISIS 2503 (5 mg/kg)					
	10	4	0.090	0.008	0.004
	14	4	0.208	0.036	0.018
	21	4	0.550	0.153	0.077
	30	4	0.998	0.345	0.173
PEG-Liposome/ISIS 2503 (25 mg/kg)					
	10	6	0.102	0.047	0.019
	14	6	0.142	0.084	0.034
	21	6	0.283	0.172	0.070
	30	6	0.603	0.331	0.135
PEG-Liposome/ISIS 2105 (25 mg/kg)					
	10	5	0.120	0.038	0.017
	14	5	0.294	0.180	0.081
	21	5	0.996	0.735	0.329
	30	5	1.508	0.981	0.439

TABLE 20

Antitumor Activity of Liposomal Formulations of ISIS 2503 Against NCI-H69 Xenografts

			Tumor Size (mm^3)		
Formulation:	Day	n	Mean	SD	Std. Error
Saline[a]/no oligonucleotide					
	21	7	0.150	0.061	0.023
	28	7	0.513	0.493	0.186
	35	7	0.749	0.392	0.148
	42	7	2.106	2.277	0.861
Saline[a]/ISIS 2503 (25 mg/kg)					
	21	7	0.163	0.056	0.021
	28	7	0.334	0.205	0.077
	35	7	0.766	0.545	0.206
	42	7	1.021	0.751	0.284
PEG-Liposome[b]/ISIS 2503 (25 mg/kg)					
	21	6	0.150	0.068	0.028
	28	6	0.222	0.121	0.049
	35	6	0.417	0.251	0.102
	42	6	0.753	0.551	0.225
PEG-Liposome[b]/ISIS 13177 (25 mg/kg)					
	21	7	0.163	0.043	0.016
	28	7	0.460	0.233	0.088
	35	7	0.956	0.410	0.155
	42	7	1.636	1.037	0.392

[a]Saline formulations given qd.

[b]liposomal formulations given q3d.

In the experiment described in Table 19, sterically stabilized liposomes comprising DPSE-MPEG$_{2000}$ and ISIS 2503 were given in doses of 1, 5 and 25 mg/kg. Controls included a saline solution (0.9% NaCl) and sterically stabilized liposomes comprising ISIS 2105. Dosing was i.v. q3d. As can be seen in Table 19, treatment with sterically stabilized liposomes comprising ISIS 2503 resulted in a dose-dependent reduction in the rate of tumor growth. At day 21, tumor sizes averaged 0.964 and 0.996 mm^3 for the animals treated with, respectively, saline and liposomal ISIS 2105. In contrast, animals treated with liposomal ISIS 2503 at 1, 5 and 25 mg/kg had tumors averaging 0.700, 0.550 and 0.283 mm^3, respectively.

A similar experiment (Table 20) shows that the liposomal oligonucleotide formulation is also effective against NCI-H69-derived xenografts. In this experiment, animals treated with 25 mg/kg of ISIS 2503 given as part of a liposomal formulation had tumors averaging 0.417 mm^3 in size on day 35, as compared to 0.749 and 0.766 mm^3 for saline alone and saline formulated oligonucleotide, respectively. Treatment with a liposomal formulation comprising a control oligonucleotide (ISIS 13177) at 25 mg/kg resulted in tumors averaging 0.956 mm^3 on day 35.

The above results demonstrate that sterically stabilized liposomal oligonucleotide formulations have several advantages over traditional formulations. First, the liposomal formulations of the invention result in improved pharmacodynamic properties (*e.g.*, prolonged clearance time from the blood, enhanced biostability in blood and kidney samples, *etc.*) that result in greater circulating concentrations and stability of full-length oligonucleotides. Second, the liposomal formulations of the invention result in enhanced delivery, relative to traditional saline formulations, of the oligonucleotides encompassed thereby to tumor tissues. Third, due at least in part to the above features, liposomal oligonucleotide formulations can achieve higher concentrations

and greater specific effects attributable to antisense oligonucleotides using a less frequent dosing regime than seen with traditional formulations (*e.g.*, as seen from the data in Table 20, 25 mg/kg of ISIS 2503 given every third day in a liposomal formulation was more effective than the same dose of ISIS 2503 given daily in saline). Taken together, these properties are expected to result in an efficacious method for treating an animal, including a human, suffering from a hyperproliferative disease or disorder such as cancer.

EXAMPLE 6: Pharmacokinetics of ISIS 2503 oligonucleotide encapsulated in stealth liposomes administered rhesus monkeys

Liposomal ISIS 2503 was formulated as 7 mg/mL oligonucleotide with a nominal lipid concentration of 100 mg/mL. ISIS 2503 was encapsulated using the thin-film hydration method. The lipids used were HSPC (hydrogenated soy phosphatidylcholine):mPeg-DSPE (N-(carbamoyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine):cholesterol at a molar ratio of 56.4:5.3:38.3. ISIS 2503 was dissolved in TE/NaCl buffer pH 7.4 at 10 mg oligo/mL buffer. Particle size of 100-110 nm was achieved by extrusion through polycarbonate membranes. Sephadex G200-120 resin with TE/NaCl buffer as eluent was used to remove the unencapsulated oligo. 25-30% encapsulation was achieved, and oligo concentration in the liposomes was determined by a solvent extraction method using phenol:chloroform:isoamyl alcohol (25:24:1, V/V/V).

Twelve rhesus monkeys (*Macaca mulatta*) (6 males and 6 females) were used in the liposome formulation group. These animals were pre-pubertal to young adult (in the age range of 3-7 years), and their body weight ranged from 3-4 kg. Each animal received a single intravenous infusion of ISIS 2503 encapsulated in liposomes (10 mg/kg) over approximately 30

minutes. Blood samples for pharmacokinetic analysis were collected prior to dosing and at 0, 1, 2, 6, 12, 24, 40, 60, 96, 120, 144, 168, 192, 240, 384 and 576 hours after dosing. Two animals (1 male and 1 female) were serially euthanized at each of the following time points from the end of infusion: 24, 60, 120, 168, 384 and 576 hours. A full necropsy was conducted on all animals. The following tissues were collected from each animal: brain, heart, pancreas, prostate, ovaries, spleen, intestine, kidney cortex, kidney medulla, liver, mesenteric and mandibular (combined, M & M) lymph nodes, axillary and inguinal (combined, A & I) lymph nodes, lung, back skin, and hand skin. Both whole blood and tissue samples were extracted and analyzed by capillary gel electrophoresis.

In a separate study, six rhesus monkeys (3 males and 3 females) received a 2-hour i.v. infusion of 10 mg/kg ISIS 2503 formulated in saline solution every-other-day for 28 days. Blood samples were collected in EDTA tubes pre-dose, at 1 and 2 hrs during infusion, and at 1, 2 and approximately 24 hrs after the end of the infusion of the first dose. Monkeys were sacrificed 48 hours after the last dose. The following tissues were collected from each animal: heart, pancreas, spleen, kidney cortex, kidney medulla, liver, axillary and inguinal lymph nodes, lung, colon, bone marrow, prostate, ovaries, and uterus. Both plasma and tissue samples were extracted and analyzed by capillary gel electrophoresis.

Prior to sample extraction, an appropriate amount of the internal standard (T_{27}, a 27-mer phosphorothioate oligodeoxythymidine) was added to every sample. Blood samples were extracted first with phenol-chloroform. The oligonucleotides were extracted in the aqueous phase. The aqueous phase was then evaporated to dryness, re-suspended with 5 mL strong anion exchange (SAX) loading buffer (containing 10 mM Tris-HCl, 0.5 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction. Samples for solid

phase extraction were processed identically as described by Leeds et al (*Anal. Biochem.*, *235*:36, 1996). Plasma samples (100 µL) were diluted with 5 mL SAX loading buffer followed by solid phase extraction as described with the following exceptions: the KCl concentration was decreased from 0.5 M to 0.25 M in the strong anion exchange running buffer, the reverse-phase solid phase extraction column used was the Glen Research Poly-Pack™ column (Glen Research, Sterling, VA) in place of the Isolute reverse-phase column, and purified oligonucleotide was eluted with 3 mL of freshly prepared 50% acetonitrile rather than 20% acetonitrile.

The method for extracting oligonucleotide from the tissues of monkeys treated with liposome formulated ISIS 2503 combined the proteinase K digestion method previously used for extraction of oligonucleotides from tissues with solid phase extraction method (Leeds et al, supra). Monkey tissues were weighed, homogenized in a Bio Savant (Bio 101, Inc., Vista, CA), and incubated for 24 hours at 37°C in a 2.0 mg/mL proteinase K (Activity: 30 units/mg, Boehringer Mannheim, Germany) solution of digestion buffer consisting of 0.5% Nonidet P-40 (NP-40) with 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 100 mM NaCl. An appropriate amount of T_{--} was added for quantitation by capillary electrophoresis. The aqueous layer was then extracted with phenol-chloroform, the phenol-chloroform layer was back-extracted with 500 µL of water and the aqueous phases were pooled. The aqueous layer was extracted again with chloroform to remove the phenol. Samples were then evaporated to dryness, resuspended in 200 µL concentrated ammonium hydroxide and incubated at 55°C for 12 to 24 hours. The samples were then re-evaporated to dryness, re-suspended with 5 mL SAX loading buffer (containing 10 mM Tris-HCl, 0.25 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction. Samples for solid

phase extraction were processed identically as previously described.

The method used for the extraction and analysis of the tissue samples from tissues of monkeys treated with the saline formulation was a modified procedure from that previously described, wherein proteinase K digestion and ammonium hydroxide incubation steps were omitted, and phenyl columns were used for solid phase extraction.

A Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments, Irvine, CA) was used for gel-filled capillary electrophoresis analysis. Samples were electrokinetically injected using an applied voltage between 3-10 kV for a duration ranging from 3-20 seconds. Length-based separation of the oligonucleotides was achieved by using a coated-capillary (Bio-Rad Laboratory, Hercules, CA) with Beckman eCAP ssDNA 100-R Gel (Beckman Instruments, Fullerton, CA). Separation was optimized using a constant applied voltage of 20 kV and a 40°C temperature. Oligonucleotide peaks were detected by UV absorbance at 260 nm. Beckman System Gold Software on the P/ACE instrument was used to determine the areas under the curve for oligonucleotide peaks. A peak area threshold of 0.01 area units and minimum peak width of 0.08 min were the standard integration parameters.

Quantitation of intact ISIS 2503 and metabolites for whole blood samples was based on the calibration curve with T_{27} as the internal standard. The limit of quantitation for this assay has been estimated to be 0.10 µg/mL ISIS 2503 in blood.

In contrast, the concentrations of ISIS 2503 and metabolites in the plasma and tissue samples were calculated from the ratio of the absorbances, based on the starting concentration of internal standard (T_{27}) added to the samples using the following equation:

$$C_2 = C_1(E_1/E_2)[(A_2/T_{m2})/(A_1/T_{m1})]$$

wherein C_1=concentration of the internal standard, C_2=concentration of the analyte (ISIS 2503 or metabolites), E_1=molar extinction coefficient of the internal standard, E_2=molar extinction coefficient of the analyte, A_1=area of the internal standard peak, A_2=area of the analyte peak, T_{m1}=migration time of the internal standard peak, and T_{m2}=migration time of the analyte peak. Calculations of extinction coefficients for ISIS 2503, metabolites, and T_{27} were made using a program that calculates the sums of the extinction coefficients from the individual bases according to the base composition. For the calculation of extinction coefficients, metabolites are assumed to be generated by loss of nucleotide from the 3'-end. The limit of quantitation for this assay has been estimated to be 0.07 µg/mL in plasma and 0.10 µg/g in tissue.

In triplicate, encapsulated and unencapsulated ISIS 2503 (1 µM) were added to 1 mL of monkey blood, respectively. After being centrifuged at 1500 rpm (2000 *g*) for 10 minutes, the upper plasma layer was separated from the red blood cell (RBC) layer. An aliquot of 100 µL from each layer was extracted and analyzed using the method described above. T_{27} (5 µM) was added to each sample prior to sample extraction as internal standard.

The study of ISIS 2503 (both encapsulated and unencapsulated) distribution in blood indicated no binding or distribution of ISIS 2503 (both encapsulated and unencapsulated) on or in the red blood cells. Therefore, concentrations of ISIS 2503 in plasma were calculated from concentrations in blood by correcting for hematocrit values which for the purposes of this calculation were assumed to be 0.41. The following equation was used:

[ISIS 2503 Conc. in plasma]=[ISIS 2503 Conc. in blood]/(1-hematocrit)]

Inspection of the semi-logarithmic plots of intact ISIS 2503 (full length) plasma concentration-versus time curves indicated that they could be described by a monoexponential equation. First order elimination was assumed. Initial estimates of parameters were obtained by linear regression of the terminal concentration time points. Nonlinear regression was accomplished using a one compartment model for each individual animal (WinNonlin 1.0). A uniform weight of 1 was used for all plasma concentration data. Four of the animals were excluded from complete individual pharmacokinetic analysis of plasma concentrations because they were sacrificed before a complete plasma profile could be collected (2 at 24 hr and 2 at 60 hr).

Tissue elimination was analyzed by noncompartmental methods using WinNonlin 1.0. Tissue half-lives were estimated by linear regression analysis of the log-linear terminal phase of the tissue concentration-time curve. The area under the tissue concentration-time curve ($AUC_{0-\infty}$) and the area under the first moment of the concentration-time curve ($AUMC_{0-\infty}$) were calculated using the linear trapezoidal rule, up to the last measured time point, plus the extrapolated area. The mean residence time (MRT) was calculated as the ratio of the $AUMC_{(0-\infty)}$ to the $AUC_{(0-\infty)}$.

The plasma pharmacokinetics of ISIS 2503 formulated in the saline solution after single dose was analyzed by noncompartmental methods using WinNonlin 1.0. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule, up to the last time point, plus the extrapolated area.

Statistical analysis for gender difference of kinetic parameters was performed by F-test (Excel 6.0) for the analysis of variance, and t-test (Excel 6.0) for the analysis of mean at the $p = 0.05$ level. Descriptive statistics were used to present data summaries for pharmacokinetic parameter estimates.

The time course of the clearance of ISIS 2503 and oligonucleotide metabolites from plasma is greatly prolonged for the liposome formulation compared with the saline formulation (Table 21). Mean maximum plasma concentration (C_{max}) of intact ISIS 2503 in the liposome formulation group was approximately 172 µg/mL and was reached at the end of the 30-minute infusion. Concentrations of ISIS 2503 in plasma decreased slowly to approximately 18 µg/mL at 144 hours after infusion. In contrast, after administration of the saline formulation, mean maximum plasma concentration (C_{max}) of intact ISIS 2503 was approximately 65 µg/mL and was reached at the end of the 2-hr infusion. ISIS 2503 concentration in plasma declined four-fold 2 hrs after infusion, and was below the limit of detection at 24 hrs after infusion.

Estimates of the pharmacokinetic parameters for males and females were averaged and combined since statistical analysis indicated no significant gender differences. The mean half-life in plasma for the liposome formulated ISIS 2503 was 57.8 hours, whereas the mean half-life in plasma for saline formulated ISIS 2503 was only 1.07 hours (Table 22). The average total body clearance and Vd_{ss} for the liposome formulation were 0.892±0.161 mL/hr/kg and 73.0 ± 15.3 mL/kg, respectively. The average total body clearance and Vd_{ss} for the saline formulated ISIS 2503 were 54.0 ± 12.0 mL/hr/kg and 85 ± 17 mL/kg, respectively. Therefore, the total body clearance after administration of the saline formulated ISIS 2503 was over 50 times faster than after administration of the liposome formulation, indicating that ISIS 2503 in the liposome formulation was distributed and metabolized at a much slower rate compared with the saline formulation.

For the liposome formulation group, there were very small amounts of oligonucleotide metabolites observed in plasma (Table 21). A few species of chain-shortened metabolites cumulatively represented approximately less than 10% of the

total oligonucleotides at all time points in plasma. A low level of metabolites could be observed 60 hrs after infusion of liposomal ISIS 2503. In contrast, metabolism was much more active in the saline formulation group, where the chain-shortened metabolites represented 35 to 45% of total oligonucleotides in plasma (Table 21). Moreover, more species of metabolites were also formed. A series of metabolites resulted from progressive shortening of ISIS 2503 by nucleases were observed 1 hr after the beginning of infusion.

After administration of ISIS 2503 in the liposome formulation, ISIS 2503 was distributed widely into all the tissues analyzed. The highest tissue concentrations of total oligonucleotide were measured in liver, with slightly lower concentrations detected in spleen, followed by the lymph nodes, lung, hand skin, kidney cortex and medulla, heart, back skin, pancreas, colon and brain. It appears that the primary organs of encapsulated ISIS 2503 distribution were the organs of the reticulo-endothelial system. Tissue distribution of liposomal ISIS 2503 differed from ISIS 2503 and other oligonucleotides in the saline formulations and other unencapsulated oligonucleotides studied previously, where the highest concentration of oligonucleotide was consistently observed in kidney cortex. Moreover, oligonucleotide concentration in kidney cortex was significantly higher than the concentration in kidney medulla for unencapsulated ISIS 2503, and this concentration difference was not observed in liposome formulated ISIS 2503. Relatively long half-lives of ISIS 2503 were observed in all tissues studied (Table 23) following administration of ISIS 2503 in liposomes. The mean residence time (15 days) of ISIS 2503 in the kidney cortex was the longest among all the tissues examined. Uptake was slow in all tissues with time to peak concentration from 1 - 7 days. The concentration of ISIS 2503 in brain, prostate, and ovaries was still increasing up to seven days post-dosing.

For the liposome formulation group, the appearance of metabolites was low even 576 hours after infusion. Only 10 to 20% of the total oligonucleotide was in the form of metabolites in most organs. In liver, kidney cortex, and pancreas, 30 to 60% of the oligonucleotide were metabolites. Significant amounts of oligonucleotide metabolites were observed as early as 24 hours after infusion in the pancreas. At later time points (≥ 120 hr), increasing concentrations of chain-shortened oligonucleotide metabolites were seen in liver and kidney. In comparison, a much higher percentage of metabolites (~62-84%) were observed in the tissues of the saline formulation group at 48 hrs from the last dose.

TABLE 21

Concentrations (μg/mL) of ISIS 2503 in plasma after the end of 0.5 hr (for the liposome formulation) and 2 hr (for the saline formulation) Intravenous infusion of 10 mg/kg ISIS 2503 to Rhesus Monkeys

	Liposome Formulation		Saline Formulation	
Time	ISIS 2503 (μg/mL)	%Intact	ISIS 2503 (μg/mL)	%Intact
0	151 ± 41.8	95.6	65.0 ± 12.5	61.0
1	153 ± 32.0	96.1	30.2 ± 5.7	55.3
2	139 ± 37.3	96.1	17.4 ± 4.3	55.0
6	133 ± 34.6	96.0	NA	NA
12	109 ± 31.5	97.8	NA	NA
24	107 ± 30.8	96.7	nd	nd
40	84.8 ± 25.2	96.8	NA	NA
60	85.8 ± 15.9	92.2	NA	NA
96	38.3 ± 21.8	91.9	NA	NA
120	26.6 ± 17.3	89.2	NA	NA

144	17.9 ± 10.7	94.2	NA	NA
168	10.5 ± 6.55	95.1	NA	NA
192	6.14	84.5	NA	NA
240	3.61	64.2	NA	NA
384	nd	nd	NA	NA
576	nd	nd	NA	NA

Full length % = percent of total detectable oligonucleotide represented by intact ISIS 2503.

nd = not detected, detection level = 0.07 µg/mL.

NA = not applicable (samples were not collected).

TABLE 22

Summary of estimated pharmacokinetic parameters in plasma for ISIS 2503 (10 mg/kg) encapsulated in liposomes administered to Rhesus Monkeys by 0.5 hr infusion (n=8) and in saline formulation administered to Rhesus Monkeys by 2 hr infusion (n=6)

Parameter	Liposome (Mean ± SD)	Saline Formulation (Mean ± SD)
AUC (µg*hr/mL)	1.15×10^4 ± 2.07×10^3	156 ± 30
$t_{1/2}$ (hr)	57.8 ± 13.1	1.07 ± 0.19
C_{max} (µg/mL)	172[a] ± 33	65.0 ± 12.5
Cl (mL/hr/kg)	0.892 ± 0.161	54.0 ± 12.0
Vd_{ss} (mL/kg)	73.0 ± 15.3	85.0 ± 17.0

[a] Data obtained from 12 animals

TABLE 23

Estimated tissue pharmacokinetic parameters for ISIS 2503 (10 mg/kg) encapsulated in liposomes administered to Rhesus Monkeys by 0.5 hr intravenous infusion

Tissue	$T_{1/2}$ (day)	MRT (day)	T_{max} (day)	C_{max} (µg/g)
Kidney Cortex	11	15	1	13.4
Kidney Medulla	5.6	8.2	1	14.3
Liver	4.2	8.1	7	106
A & I Lymph Node[b]	NA	18	5	68.2
M & M Lymph Node[c]	7.7	13	5	43.8
Spleen	9.7	14	5	94.0
Back Skin	3.1	7.0	7	11.1
Hand Skin	4.2	10	2	25.7
Lung	2.0	3.4	2	26.1
Heart	3.0	5.7	1	10.3
Pancreas	3.2	6.4	2	5.53
Brain	NA	NA	1	2.27
Colon	NA	NA	7[a]	6.88
Ovary	NA	NA	2	6.93
Prostate	NA	NA	7[a]	6.89

NA = not available

[a] Concentration was still increasing at the last analyzed time point.

[b] Axillary and inguinal lymph node.

[c] Mesenteric and mandibular lymph node.

What is claimed is:

1. A liposomal oligonucleotide composition comprising one or more oligonucleotides, wherein each of said one or more oligonucleotides comprises from about 8 to about 30 nucleotide units, and wherein each of said one or more oligonucleotides specifically hybridizes to a target nucleic acid, wherein said target nucleic acid is a DNA or mRNA molecule encoding a mutant or wildtype ras protein, and wherein said one or more oligonucleotides are entrapped within liposomes.

2. The composition of claim 1, wherein at least one nucleotide of at least one of said one or more oligonucleotides is modified at the 2' position of the sugar portion of said nucleotide.

3. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a chimeric oligonucleotide having a first region, wherein said first region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid, and a second region, wherein said second region forms a substrate for RNase H when hybridized to said target nucleic acid.

4. The composition of claim 3, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid is modified at the 2' position of the sugar portion of said nucleotide.

5. The composition of claim 4, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid has an alkoxy, alkoxyalkoxy of fluoro substituent at the 2' position.

6. The composition of claim 3, wherein said second region that forms a substrate for RNase H when hybridized to said target nucleic acid comprises at least one 2'-deoxynucleotide.

7. The composition of claim 1, wherein at least one of said one or more oligonucleotides has one or more phosphorothioate linkages.

8. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a peptide nucleic acid.

9. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a chimeric oligonucleotide having a first flanking region, wherein said first flanking region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid, a central region, wherein said central region forms a substrate for RNase H when hybridized to said target nucleic acid, and a second flanking region, wherein said second flanking region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid, and wherein said nucleotides modified to enhance the affinity of said oligonucleotide for said target nucleic acid in said first flanking region and said second flanking region can be the same or different.

10. The composition of claim 9, wherein at least one of said nucleotides modified to enhance the affinity of said oligonucleotide for said target nucleic acid is modified at the 2' position of the sugar portion of said nucleotide.

11. The composition of claim 10, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for

said target nucleic acid has an alkoxy, alkoxyalkoxy or fluoro substituent at the 2' position.

12. The composition of claim 9, wherein said central region that forms a substrate for RNase H when hybridized to said target nucleic acid comprises at least one 2'-deoxynucleotide.

13. The composition of claim 9, wherein at least one of said one or more oligonucleotides has one or more phosphorothioate linkages.

14. The composition of claim 1, wherein at least one of said oligonucleotides is specifically hybridizable with a translation initiation site or codon 12 of the ras gene.

15. The composition of claim 1, wherein one of said oligonucleotides is ISIS 2503 having the sequence of SEQ ID NO: 2.

16. The composition of claim 1, wherein said liposomes are sterically stabilized liposomes.

17. The composition of claim 16, wherein said sterically stabilized liposomes have a mean particle size of from about 90 to about 110 nm.

18. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one underivatised vesicle-forming lipid and at least one vesicle-forming lipid which is derivatised with a hydrophilic polymer.

19. The composition of claim 18, wherein said hydrophilic polymer is a moiety comprising polyethylene glycol.

20. The composition of claim 18, wherein said hydrophilic polymer comprises from about 1 mol% to about 20 mol% of the total lipid content of the liposomes.

21. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one underivatised vesicle-forming lipid and at least one vesicle-forming phospholipid having an amino group.

22. The composition of claim 21, wherein said vesicle-forming phospholipid having an amino group is selected from the group consisting of phosphatidylethanolamine and distearoyl phosphatidylethanolamine.

23. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one sterol.

24. The composition of claim 23, wherein said sterol is cholesterol.

25. The composition of claim 1, wherein said liposomes comprise at least one lipid having two hydrocarbon chains and a polar head.

26. The composition of claim 25, wherein said lipid having two hydrocarbon chains and a polar head is selected from the group consisting of phosphatidylcholine, distearoyl phosphatidylcholine and dipalmitoylphosphatidylcholine.

27. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one glycolipid.

28. The composition of claim 27, wherein said glycolipid is ganglioside GM_1 or sphingomyelin.

29. A method of modulating the expression of a ras gene comprising contacting cells, tissues, organs or organisms expressing said ras gene with the composition of claim 1.

30. A method of inhibiting the growth of cells comprising contacting said cells with the composition of claim 1.

31. The method of claim 30, wherein said ras gene is a human ras gene.

32. The method of claim 31, wherein said human ras gene is H-ras, K-ras or N-ras.

33. A pharmaceutical composition comprising the composition of claim 1, wherein said ras gene is a mammalian ras gene.

34. A pharmaceutical composition comprising the composition of claim 1, wherein said ras gene is a human ras gene.

35. The pharmaceutical composition of claim 34, wherein said human ras gene is H-ras, K-ras or N-ras.

36. A pharmaceutical composition comprising (a) the composition of claim 1 in combination with (b) one or more

chemotherapeutic agents which do not act by an antisense mechanism.

37. The pharmaceutical composition of claim 36 in which said one or more chemotherapeutic agents is or are entrapped within sterically stabilized liposomes.

38. A pharmaceutical composition comprising the composition of claim 1 in combination with one or more non-ras-targeted oligonucleotides, wherein each of said one or more non-ras-targeted oligonucleotides comprises from about 8 to about 30 nucleotide units, and wherein each of said one or more non-ras-targeted oligonucleotides specifically hybridizes to a target nucleic acid, wherein said target nucleic acid is a DNA or mRNA molecule deriving from a mammalian mutant or wildtype cancer associated gene other than a ras gene.

39. The pharmaceutical composition of claim 38, wherein said one or more non-ras-targeted oligonucleotides is or are entrapped within sterically stabilized liposomes.

40. A method of treating or preventing a condition arising from the activation of a ras gene comprising administering to a mammal having said condition a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

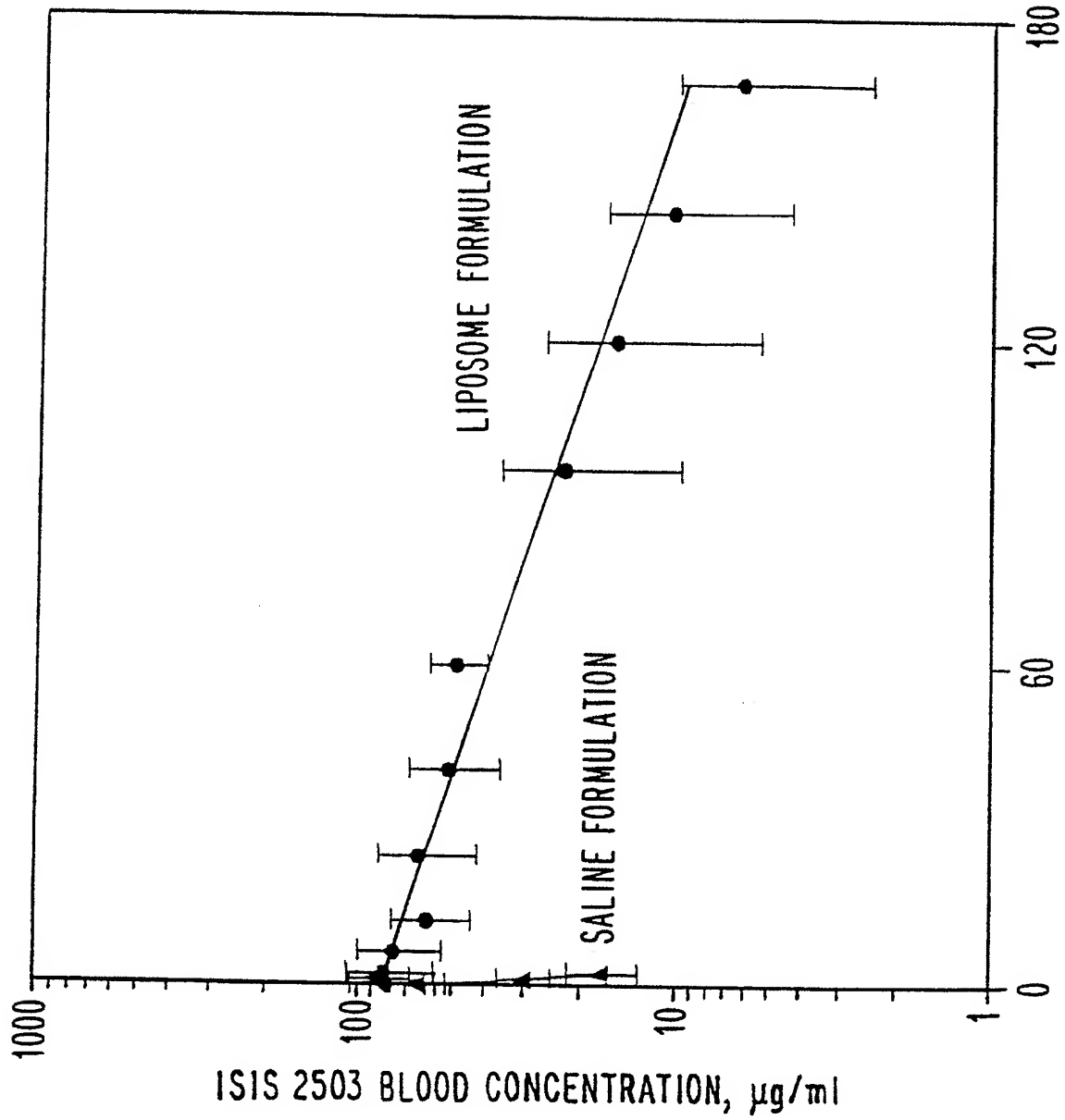
41. A method of reducing the rate of growth of a tumor or cancer in a mammal comprising administering to a mammal having a tumor or cancer a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

42. A method of treating or preventing cancer in a mammal comprising administering to said mammal a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

43. A method of treating or preventing cancer in a mammal comprising administering to said mammal a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

44. The method of claim 40, wherein said mammal is a human.

45. A pharmaceutical composition comprising the composition of claim 15.



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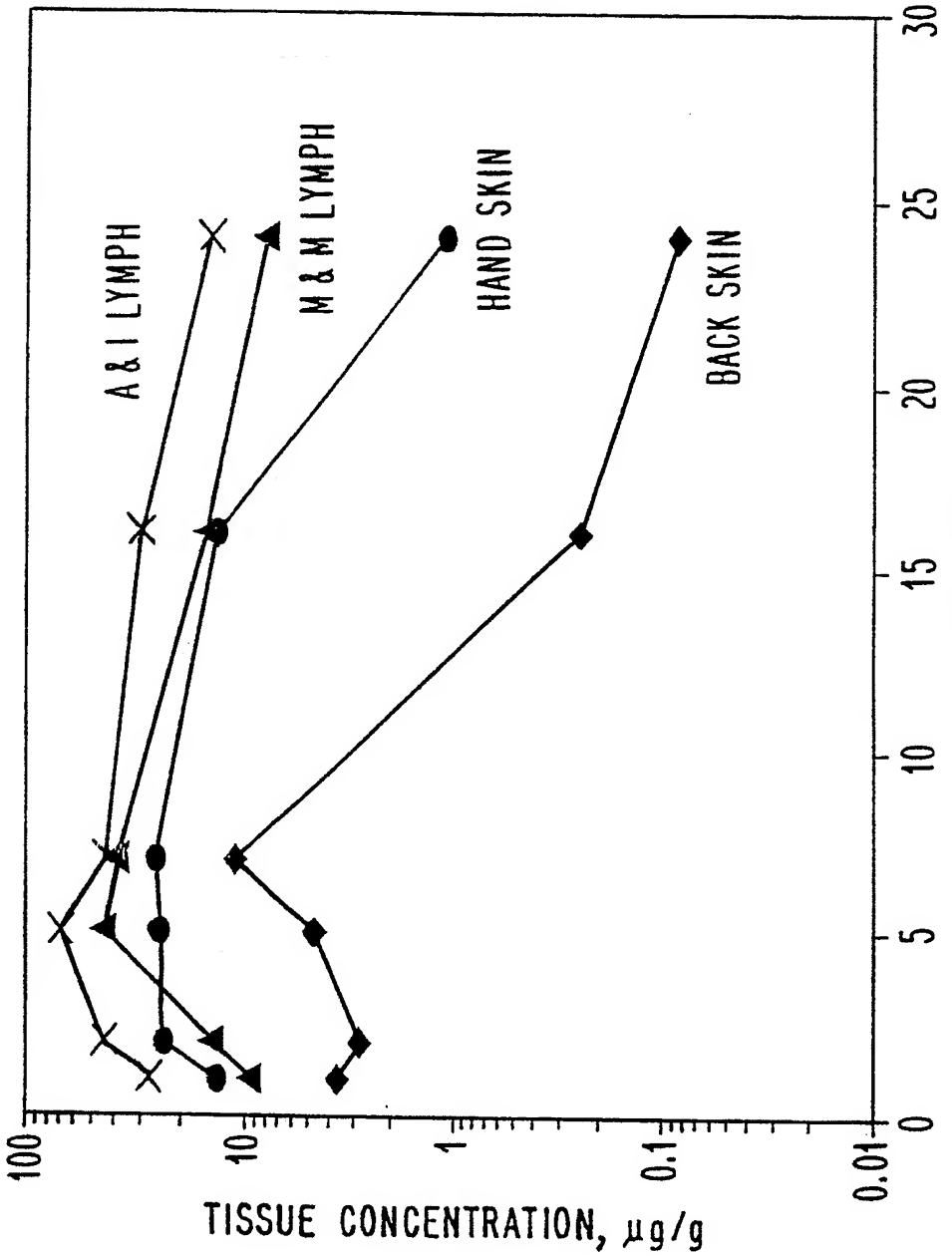

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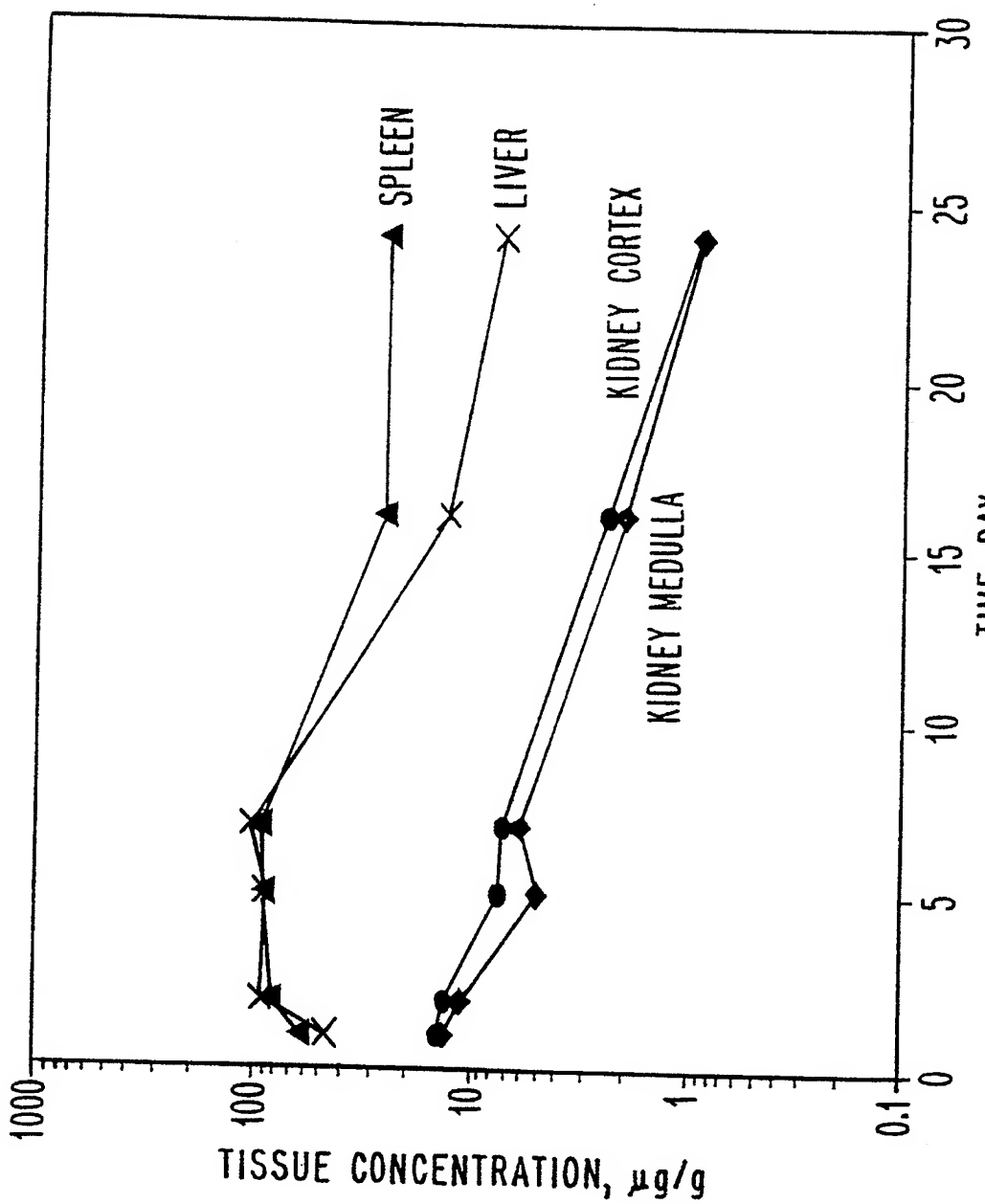

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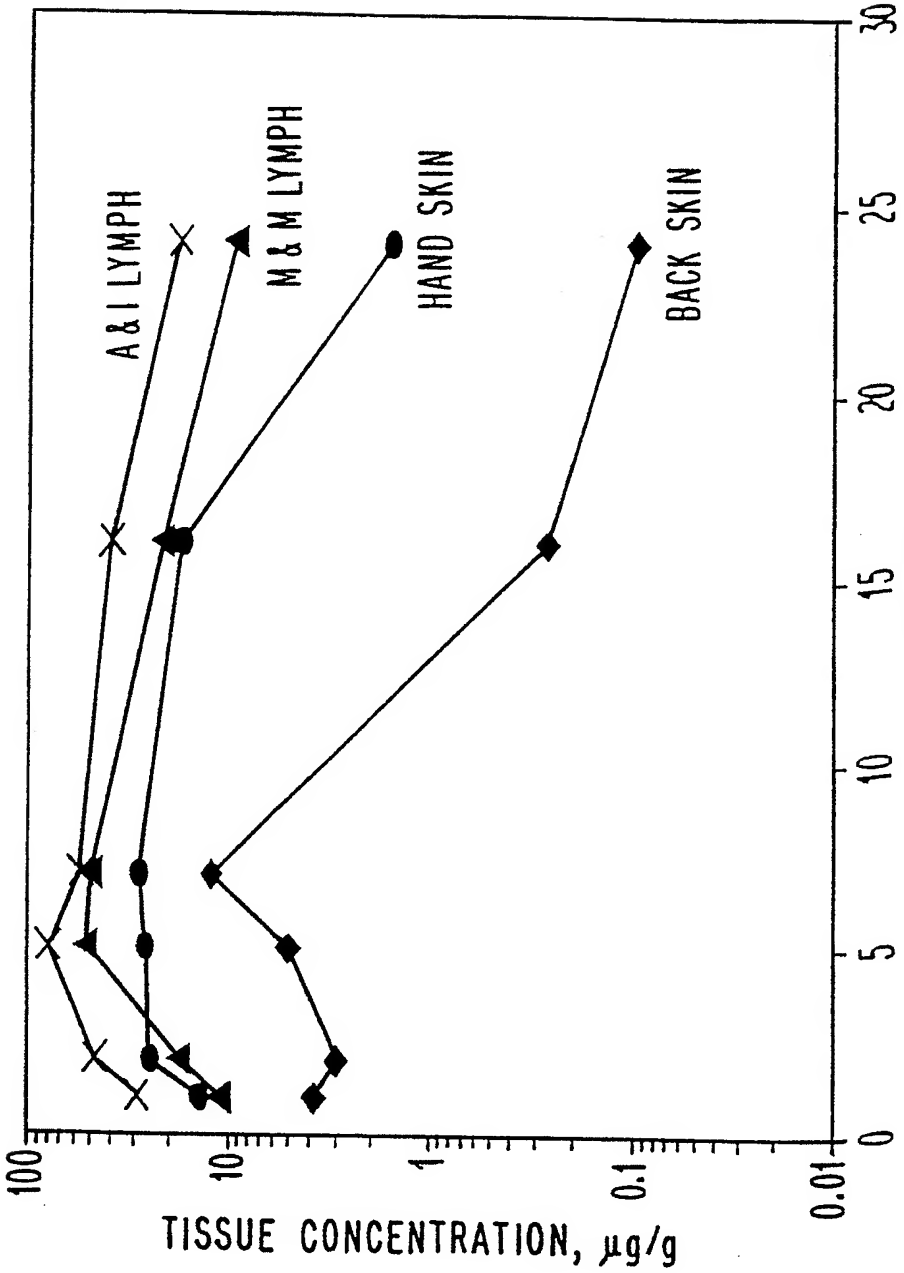
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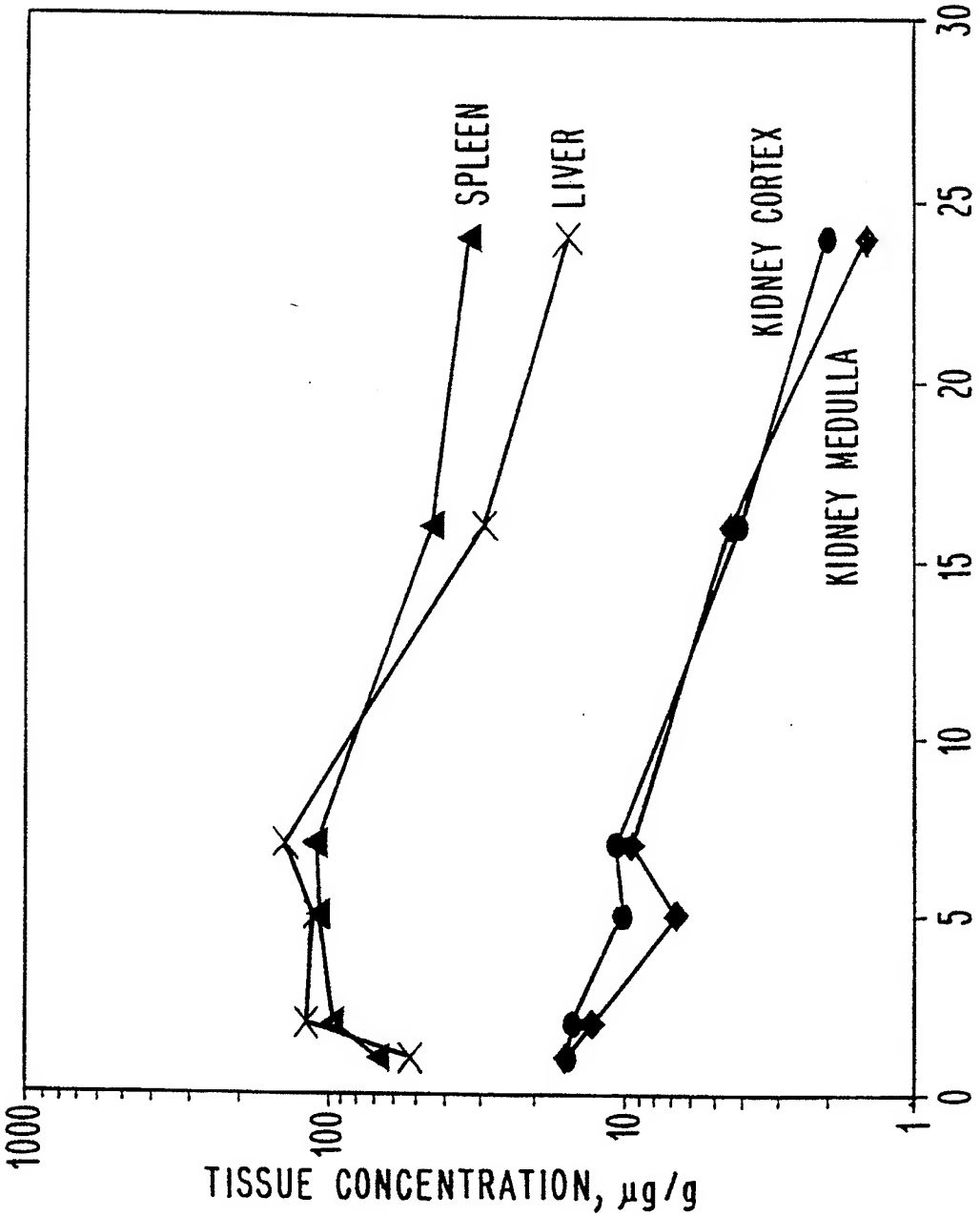
Fig. 4



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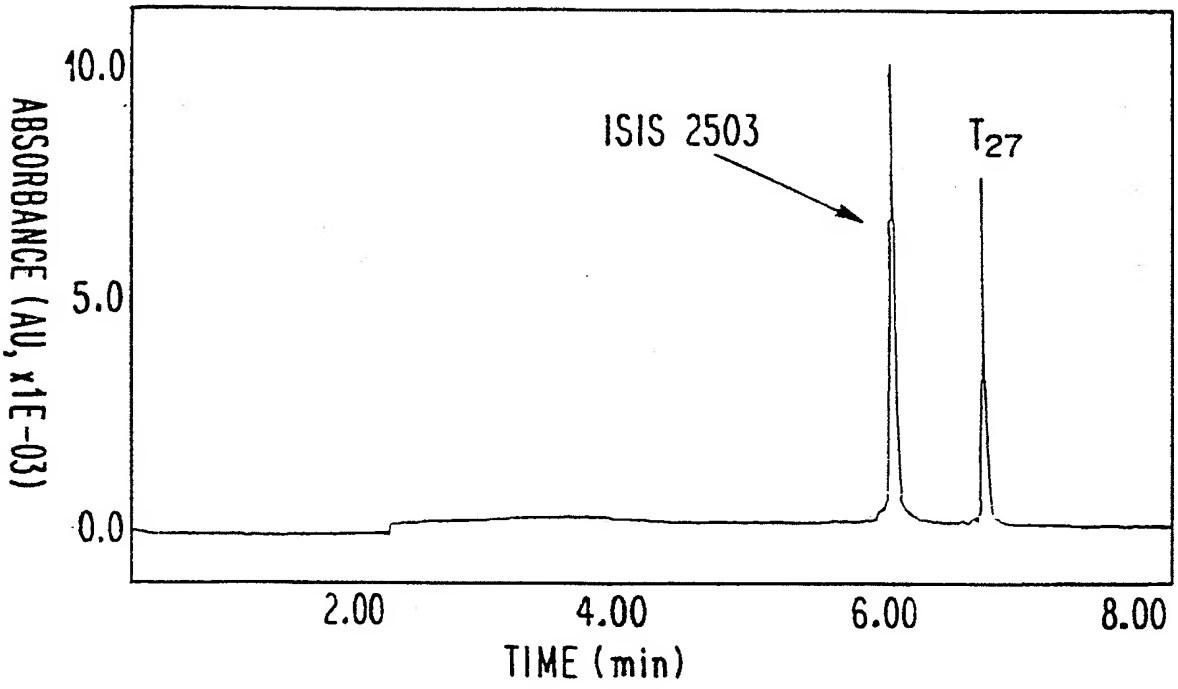
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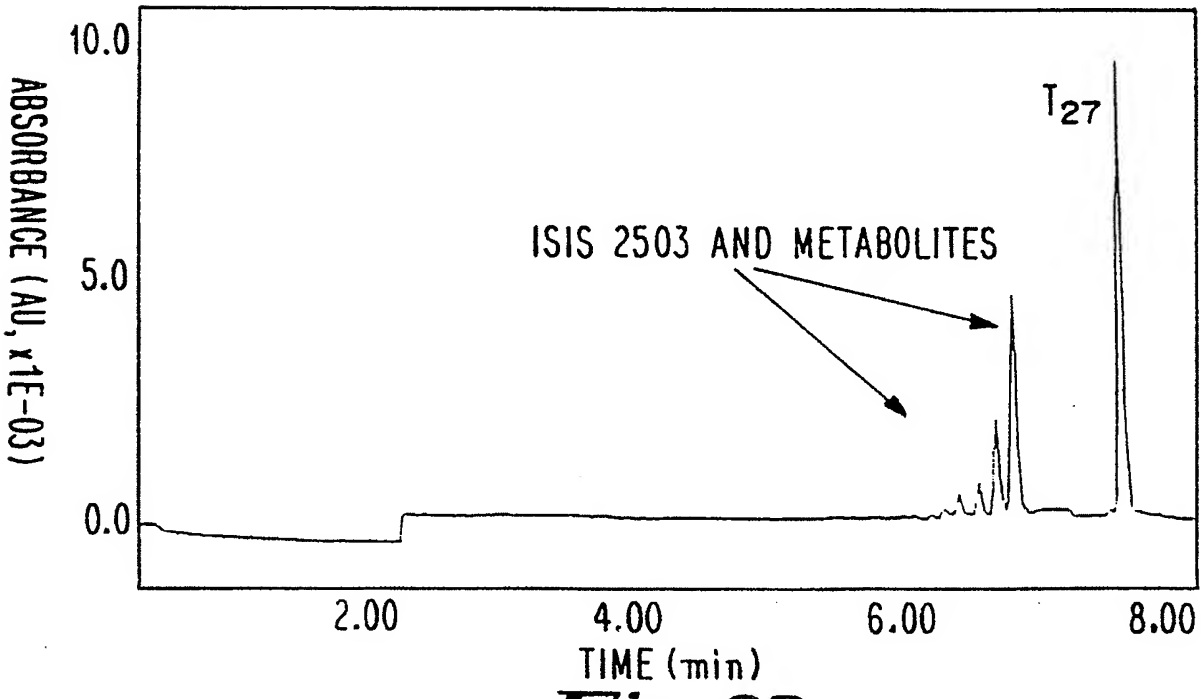
Fig. 6A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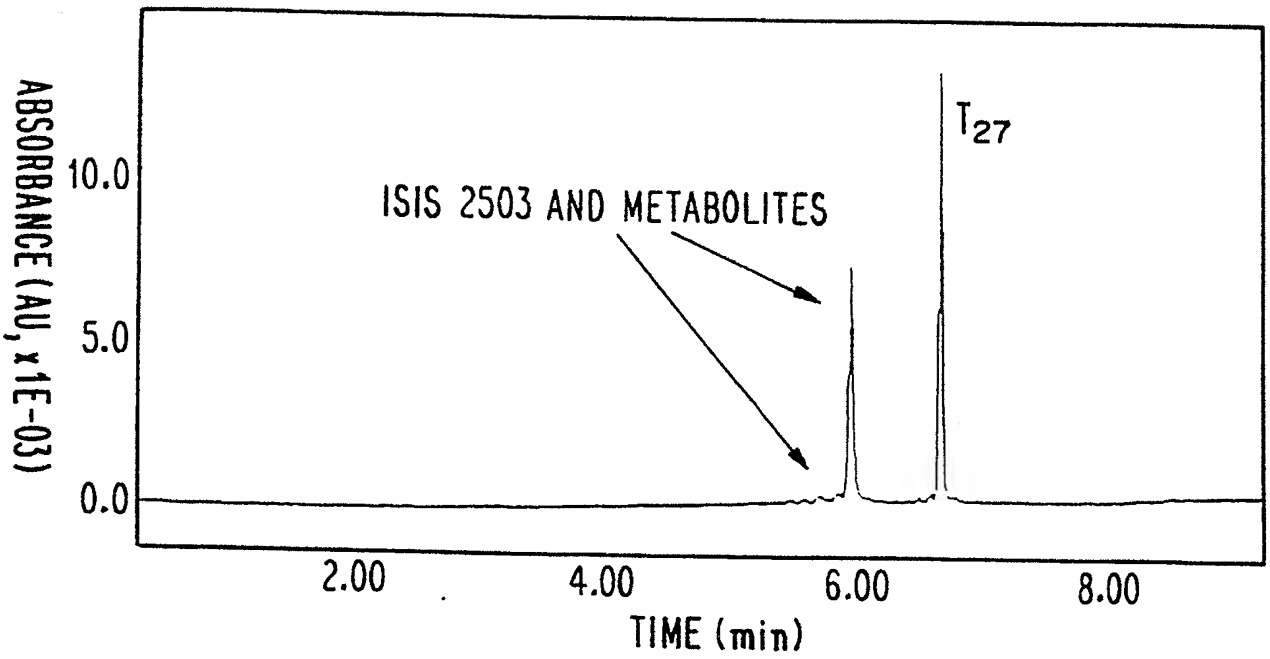

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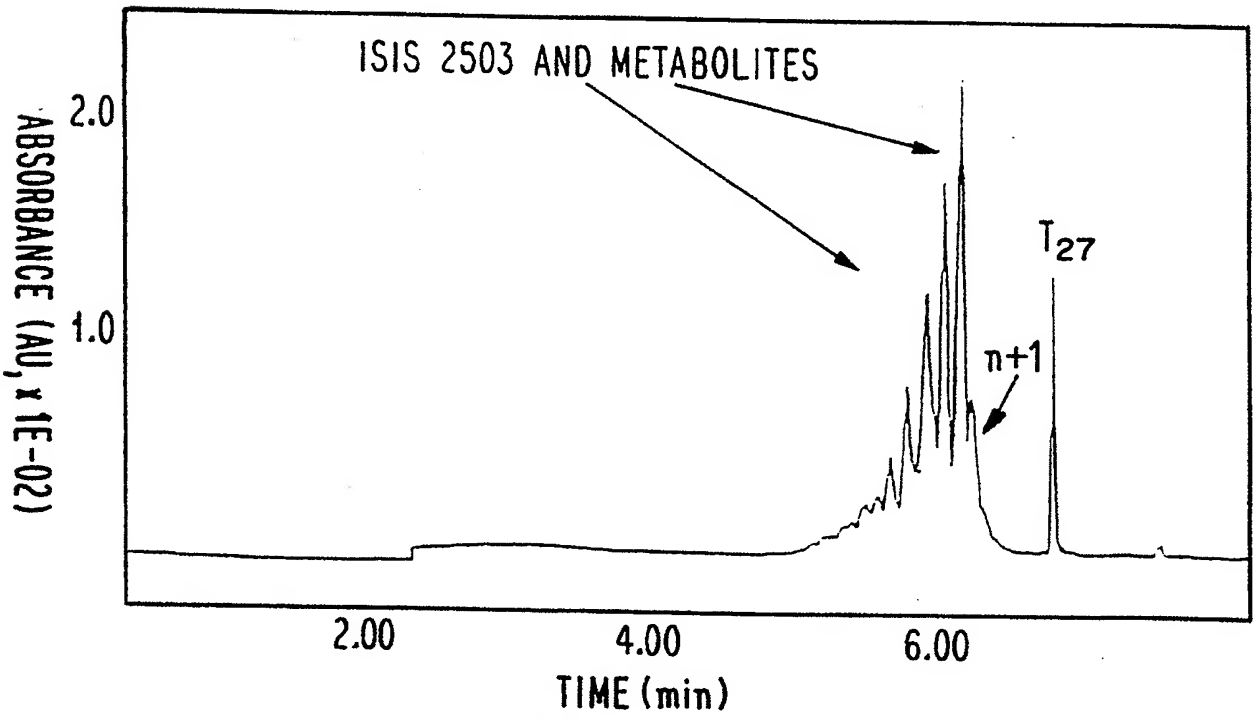

Fig. 7B

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/22821

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C07H 21/04, 21/02; C12N 15/11
US CL :536/24.1, 24.5; 435/375; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.1, 24.5; 435/375; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, SCISEARCH, EMBASE, CAPUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANG et al. Antisense Inhibition of ras p21 Expression that is Sensitive to a Point Mutation. Biochemistry, 27 August 1991 Vol. 30, No. 34, pages 8283-8286, see entire document.	1, 2, 7, 14, 17-27
Y	CHONN et al. Recent Advances in Liposomal Drug-Delivery Systems. Current Biology, 1995. Vol. 6, pages 698-708, see entire document.	1-32, 45
Y	US 5,576,208 A (MONIA et al.) 19 November 1996, see entire document.	1-32, 45
Y	UHLMANN et al. Antisense Oligonucleotides: A New Therapeutic Principle. Chemical Reviews June 1990. Vol. 90, No. 4, pages 543-584, see entire document.	1, 2, 7, 14, 17-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search
17 DECEMBER 1998

Date of mailing of the international search report
08FEB1999

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